Vascular Endothelial Growth Factor as an Autocrine Survival Factor for Retinal Pigment Epithelial Cells under Oxidative Stress via the VEGF-R2/PI3K/Akt

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Purpose. Vascular endothelial cell growth factor (VEGF) is strongly induced by oxidative stress in retinal pigment epithelial (RPE) cells, and VEGF-A is a survival factor for various cell types. This study was conducted to determine whether the autocrine VEGF signaling 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 by flow cytometry 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 and anti-VEGF-R2). The expression of VEGF-A, -R1, -R2, and soluble VEGF-R1 was determined by semiquantitative RT-PCR or Western blot analysis. Phosphorylation of VEGF-R2 was detected with immunoprecipitation and immunoblot analysis.

Results. Hydrogen peroxide–induced cell death was promoted by pretreatment with VEGF-A and anti-VEGF-R2–neutralizing antibodies, but not with 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 neutralizing antibodies against either 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 eyes with age-related macular degeneration, may influence RPE cell survival. (*Invest Ophthalmol Vis Sci.* 2010;51:1190–1197) DOI:10.1167/iovs.09-4144

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Supported by Korean Research Foundation Grant KRF-2008-331-E00208 provided by the Korean Government (Basic Research Promotion Fund, MOEHRD [Ministry Of Education and Human Resources Development]) and National Research Foundation (NRF) of Korea Grant M1AQ19, 2009-0082186 provided by the Korean Government (MEST [Ministry of Science, Education, and Technology]).

Submitted for publication June 14, 2009; revised July 31 and September 11, 2009; accepted September 18. 2009.

Disclosure: **S.H. Byeon**, None; **S.C. Lee**, None; **S.H. Choi**, None; **H.-K. Lee**, None; **J.H. Lee**, None; **Y.K. Chu**, None; **O.W. Kwon**, None

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VEGF-A is a potent endothelial cell mitogen, and recent studies have shown that it acts as an autocrine growth and survival factor in VEGF-A-producing cells.[1–4] Substantial evidence indicates that it 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 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; sVEGF-R1). RNA interference (RNAi) has recently emerged as a potential 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 under way.[3,9,10] Another method of blocking the VEGF-A signal is to employ a receptor tyrosine kinase (RTK) inhibitor to interrupt the 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 the retinal pigment epithelial (RPE) cells. Tonic VEGF-A expression in the RPE may be trophic for the choriocapillaries and is possibly necessary for maintenance of the choriocapillaris fenestrae.[6,11] However, VEGF-A levels are significantly higher in patients with neovascular AMD than in healthy control subjects, 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).[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 inflammation, provoking proangiogenic 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 the 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.

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	Forward	5′-GTAGCTGGCAAGCGCTCTTACCGGCTC-3′	316
	Reverse	5′-GGATTTGTCTGCTGCCCAGTGGGTAGAGA-3′	316
mbVEGF-R1	Forward	5′-CCA CCT TGG TTG CTG AC-3′	587
	Reverse	5′-TGG AAT TCG TGC TGC TTC CTG GTC C-3′	587
sVEGF-R1	Forward	5′-CCA GGA ATC ACA CAG G-3′	393
	Reverse	5′-CAA CAA ACA CAG AGA AGG-3′	393
VEGF-R2	Forward	5′-TCT GGT CTT TTG GTG TTT TG-3′	497
	Reverse	5′-TGG GAT TAC TTT TAC TTC TG-3′	497
GAPDH	Forward	5′-GCC AAG GTC ATC CAT GAC AAC-3′	511
	Reverse	5′-GTC CAC CAC CCT GTT GCT GTA-3′	511

sVEGF-R1, soluble *VEGF-R1; mbVEGF-R1,* membrane-bound *VEGF-R1.*

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 patients with neovascular AMD.[6] Also, RPE tears and choroidal atrophy in specimens from patients with treated AMD 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 signal the presence of functional VEGF-A receptors on human RPE cells.[20–25] Thus, we were of the view that an investigation of the relationship between VEGF-A expression and RPE cell activities, especially under conditions of oxidative stress, would help to explain the pathogenesis of exudative or dry AMD.

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

MATERIALS AND METHODS

Chemical Reagents and Cell Culture Medium

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 $VEGF_{165}$ (rhVEGF) from R&D Systems, Inc. (Minneapolis, MN); recombinant PlGF (placental growth factor, P1588) from Sigma-Aldrich (St. Louis, MO); anti-VEGF neutralizing antibodies (PC315) and LY294002 (440202) and SU5416 (676487) from Calbiochem (San Diego, CA); and horseradish peroxidase (HRP)–conjugated secondary antibody from Dako (Glostrup, Denmark).

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; Invitrogen-Gibco, Carlsbad, CA). The ARPE-19 cells were used within 10 passages. They were plated in six-well plates at 1.5×10^5 cells per well and incubated at 37°C under 5% (vol/vol) CO_2 to reach 70% confluence before exposure to H_2O_2. They were serum starved before H_2O_2 exposure and then treated with H_2O_2 for 16 hours, to induce oxidative stress, before they were harvested for cell death analysis.

Flow Cytometric Analysis of Apoptosis

The 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. 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 (flow cytometry; BD Biosciences). The number of gated cells was plotted on a dot plot with reference to both annexin V and PI staining.

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, sVEGF-R1, membrane-bound (mb)VEGF-R1, and VEGF-R2 were designed from available human gene sequences (Table 1).

Western Immunoblot Analysis

Adherent cells were washed with ice-cold PBS and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% glycerol [vol/vol], 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% Triton X-100 [vol/vol]; Sigma-Aldrich) on ice for 30 minutes. Lysates were sonicated and centrifuged for 10 minutes at 12,000*g*, and the cell homogenate fractions were stored at −70°C until used.

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% (wt/vol) 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.

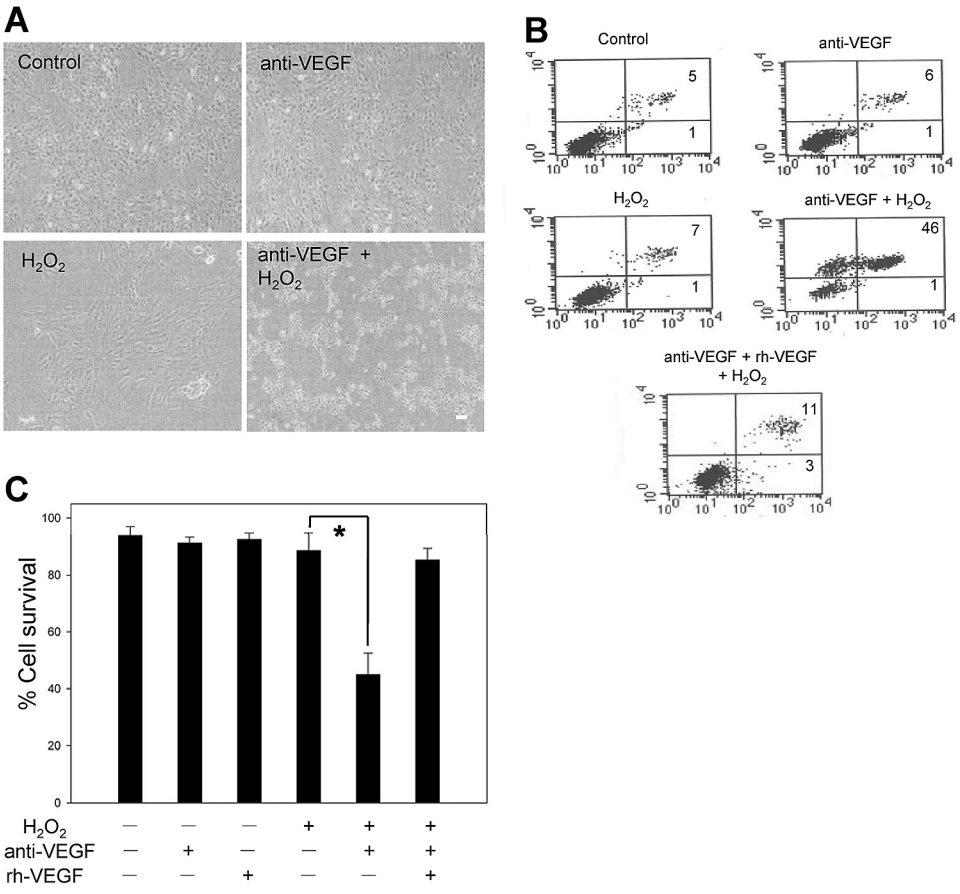

FIGURE 1. Autocrine VEGF-A protected against H_2O_2-induced cell death in ARPE-19 cells. (**A**) Immortalized ARPE-19 cells were cultured with 10% fetal bovine serum and DMEM:F12 medium. When the cells were 70% confluent, anti-VEGF antibody was treated 2 hours before treatment with 300 μM of H_2O_2. After 16 hours of H_2O_2 treatment, photographs were taken by inverted microscopy. Bar, 100 μm. (**B**) ARPE-19 cells were incubated with 200 μM H_2O_2 for 16 hours, and the cells were next analyzed by using annexin V-fluorescein isothiocyanate and PI staining. Each panel shows a typical flow cytometric histogram of 10,000 cells/sample from a representative experiment. LL, viable and undamaged cells (annexin V^-, PI^-); RL, cells undergoing early apoptosis (annexin V^+, PI^-); RU, necrotic or late apoptotic cells (annexin V^+, PI^+). (**C**) ARPE-19 cells were incubated with 200 μM H_2O_2 for 16 hours. Cell survival was then analyzed by flow cytometry. Each bar shows the mean ± SD of results in 9 to 12 wells in three independent experiments. $^*P < 0.001$ compared with control.

Immunodetection of VEGF-R2 Phosphorylation

After overnight serum starvation, an equal number 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). rhVEGF (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, Santa Cruz, CA), and reprobed with antiserum to VEGF-R2. Protein expression was quantified by densitometry.

Immunocytochemistry

Cells were fixed for 5 minutes in 3.7% (vol/vol) formaldehyde and permeabilized with 0.5% (vol/vol) Triton X-100 for 8 minutes. Single- or double-labeled immunofluorescence analysis was performed. In control experiments, the samples were run without primary antibody or after addition of an irrelevant IgG, to assess nonspecific binding of secondary antibody. In all experiments, the samples were incubated with anti-VEGF-R1 or anti-VEGF-R2 antibody for 2 hours at room temperature, followed by a 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).

Enzyme-Linked Immunosorbent Assay

Cells were treated with various concentrations of H_2O_2 at baseline (0 hours) and at 16 hours. The supernatants were collected, centrifuged to remove cell debris, and stored at −70°C before ELISA (R&D Systems), performed 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 ELISA kit (Quantikine; R&D Systems).

RESULTS

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 is related to stress-induced VEGF-A synthesis.[4,30] Pretreatment of VEGF-A–neutralizing antibodies to culture medium inhibited the ability of RPE cells to survive oxidative stress caused by H_2O_2 (Figs. 1A, 1B). Furthermore, apoptosis of RPE cells under oxidative stress conditions was inhibited by concomitant supplementation with rhVEGF (Fig. 1C). This result indicates that autocrine VEGF-A-mediated survival signals prohibit entry into the cell death pathway under conditions of oxidative stress.

Expression of VEGF-A, -R2, and -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 (Fig. 2B). Gene expression analysis indicated that expression of all VEGF-A, -R1, and -R2 was induced by H_2O_2 (Fig. 2C). Immunohistocytochemistry showed that both VEGF-R1 and -R2 protein expression was induced by H_2O_2 stimulation (Fig. 2D).

Mediation of the Autocrine VEGF-A Cell Survival Effect by the VEGF-A/VEGF-R2 Axis

Two high-affinity VEGF-A receptors, VEGF-R1 and -R2, are membrane-spanning receptor tyrosine kinases that bind

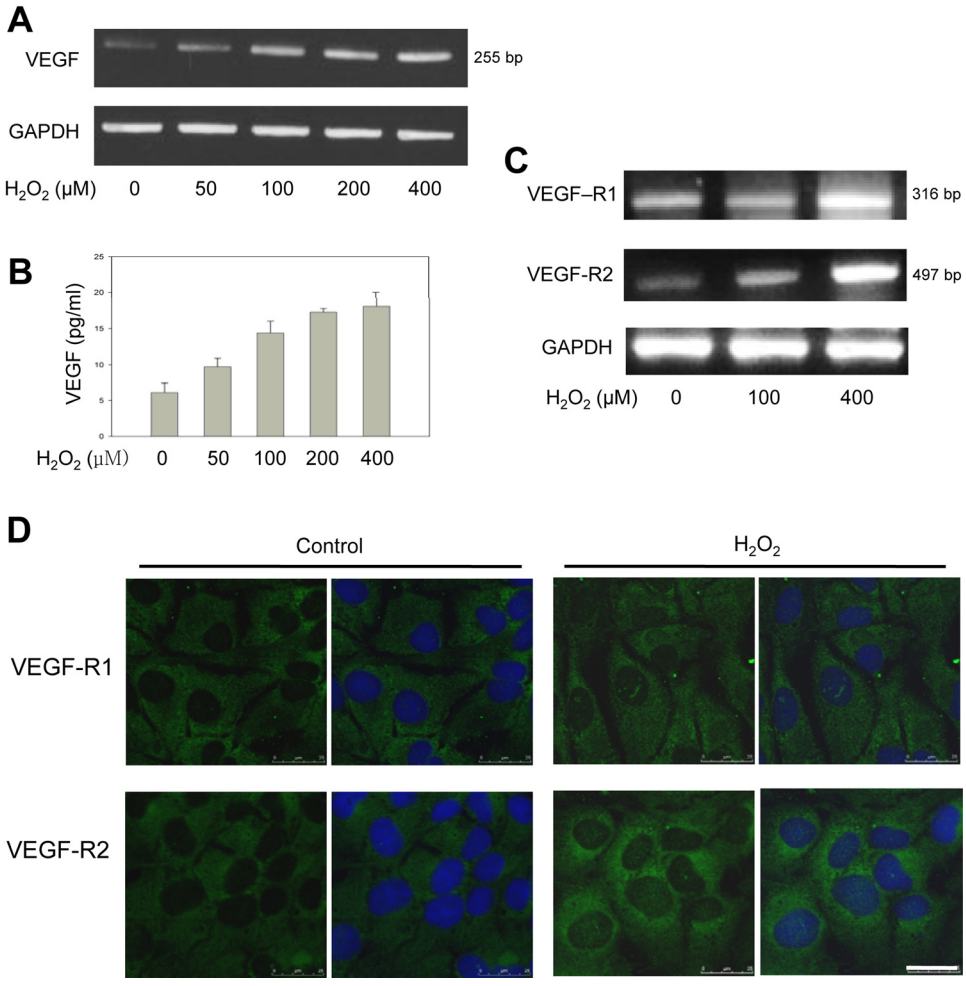

FIGURE 2. Expression of VEGF-A and VEGF receptors by H_2O_2 in ARPE-19 cells. (**A**) After 1 hour of H_2O_2 treatment, VEGF-A mRNA expression in APRE-19 cells was determined in a dose-dependent manner. (**B**) VEGF-A excretion into the medium was measured by ELISA. After 16 hours of treatment with H_2O_2, supernatant was collected and analyzed by ELISA. Data are expressed as the mean ± SD of the results in three independent experiments. (**C**) VEGF-R1 and -R2 mRNA expression was determined after H_2O_2 treatment. Each mRNA level was measured 1 hour after inoculation of various concentrations of H_2O_2. (**D**) Expression pattern of VEGF-R1 and -R2 was investigated by immunocytochemical staining. The 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 a 1-hour incubation with FITC-conjugated secondary antibody. The images were obtained with a confocal microscope. *Green*: VEGF-R1 or R2; *blue*: DAPI. Bar, 25 μm.

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 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. We found that of the two high-affinity VEGF-A receptors, VEGF-R1 and -R2, only VEGF-R2 mediated the cell survival signals. H_2O_2-induced cell death was promoted by pretreatment with anti-VEGF-R2–neutralizing antibody, but not with the use of anti-VEGF-R1–neutralizing antibody (Figs. 3A, 3B). Unlike the situation with VEGF-A, which is a ligand for both VEGF-R1 and -R2, PlGF binds only to VEGF-R1, not to VEGF-R2. In a previous result, H_2O_2-induced cell death was inhibited by supplementation with rhVEGF, but PlGF did not prevent the cell death caused by anti-VEGF-A–neutralizing antibody (Fig. 3D).

The phosphorylation levels of VEGF-R2 were measured by immunoblot analysis 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 increase on pretreatment with anti-VEGF-A antibody (Fig. 4B). 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.

The Autocrine VEGF-A Axis Influence on the Phosphorylation of the Akt Signal Protein

In RPE cells, it has been reported that the PI3k-Akt pathway stimulated by H_2O_2 is involved in protection against oxidant-induced cell death in both normal conditions and disease states such as AMD.[12] The PI3K/Akt pathway has been proposed to be activated in a 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 influences 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 the cell lysates. H_2O_2-induced phosphorylation of Akt was abrogated by pretreatment with A-neutralizing antibody against VEGF-A or SU5416 (Figs. 5A, 5C). The data thus suggest that the VEGF-A/VEGF-R2/PI3K/Akt pathway activation is involved in the resistance to cell death caused by H_2O_2 stress.

Soluble VEGF-R1 Regulation of the Autocrine VEGF-A Signal

It has been reported that sVEGF-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 the cell surface, but also on the concentration of the negative regulator (e.g., sVEGF-R1). Dur-

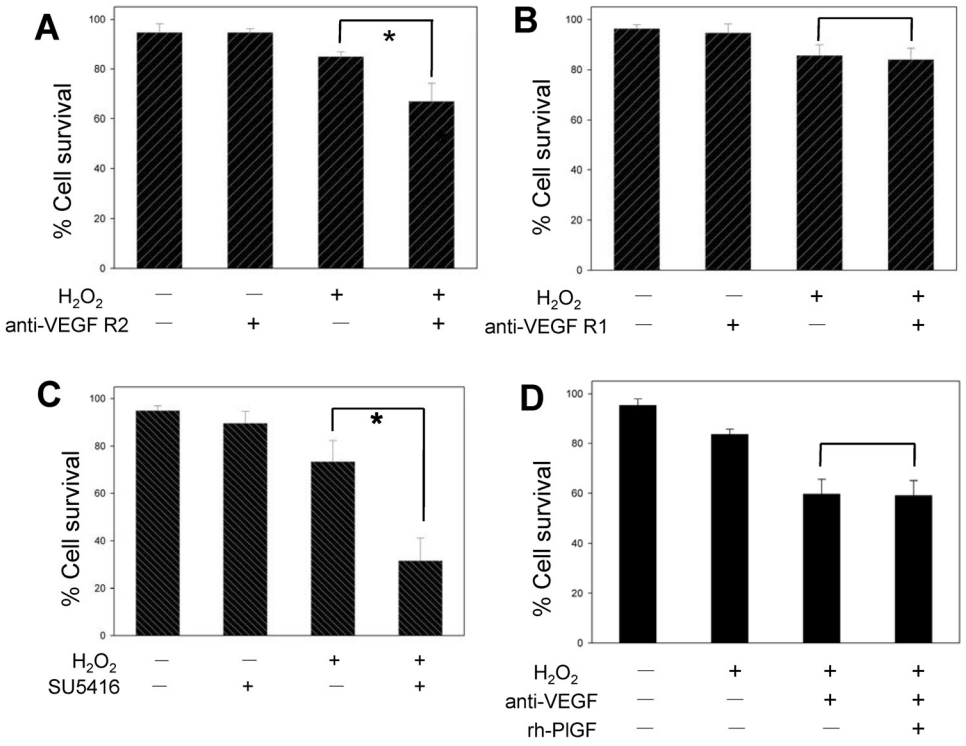

FIGURE 3. The VEGF/VEGF-R2 axis, but not that of VEGF/VEGF-R1, mediated the VEGF cell survival effect in ARPE-19 cells. Each antibody was added 2 hours before treatment with 200 μM H_2O_2 for 16 hours. Cell death was analyzed by flow cytometry of cells tagged with FITC-labeled annexin V and PI. H_2O_2-induced 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 flow cytometry showed that ARPE-19 cell survival was reduced by pretreatment with a VEGF-R2-specific PTK inhibitor (SU5416) in the presence of 300 μM H_2O_2 for 16 hours. (**D**) H_2O_2-induced cell death was promoted by pretreatment with anti-VEGF antibody, but the 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), did not prevent the cell death caused by pretreatment with anti-VEGF antibody.

ing oxidative stress, transcription of both VEGF-A and sVEGF-R1 was concomitantly induced (Fig. 6A). The transcription level of sVEGF-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 sVEGF-R1 decreased, but when VEGF-A was present at high concentrations, the sVEGF-R1 level rose (Fig. 6B).

Influence of Bevacizumab on Survival of RPE Cells under Oxidative Stress

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

DISCUSSION

The presence of functional VEGF-A receptors on RPE cells, transmitting signals similar to those mediated by receptors on endothelial cells, suggests that targeting of these receptor tyrosine kinases, either through the use of neutralizing antibody or kinase inhibitors, has 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 antileakage 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

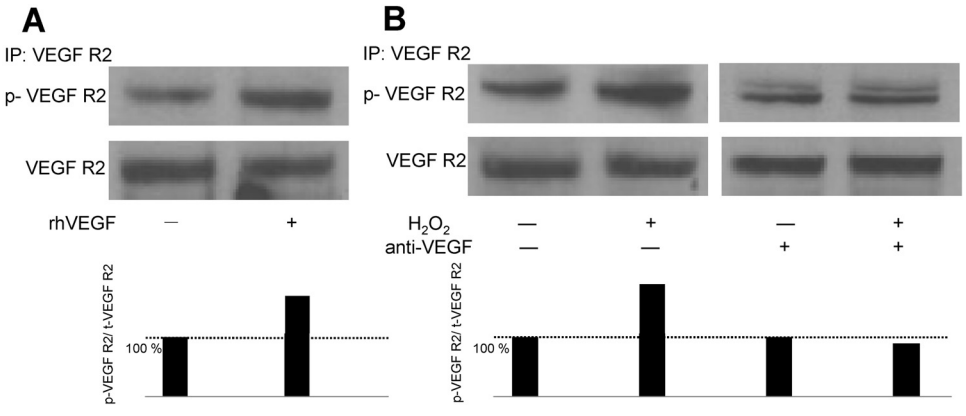

FIGURE 4. Determination of phosphorylated VEGF-R2 in APRE-19 by H_2O_2 treatment. (**A**) For positive control, phosphorylation of VEGF-R2 in ARPE-19 cells was determined by treating with rhVEGF. A nearly confluent monolayer of ARPE-19 cells was treated with 20 ng/mL of VEGF-A in serum-free medium for 24 hours. Then the cells were collected and lysed by protein lysis buffer. Immunoprecipitation was performed 400 μg of cell lysate with using 1 μg of anti-VEGF-R2 and 40 μL of protein G Sepharose. Immunoblot analysis 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. The cells were treated with 800 μM of H_2O_2 for 15 minutes and lysed with cell lysis buffer. Immunoprecipitation and immunoblot analyses were then performed. There were differences in loading of VEGF-R2, the phosphor-VEGF-R2/total VEGF-R2 levels, as follows: *lane 1*: 100%; *lane 2*: 190%; *lane 3*: 100%; *lane 4*: 90%.

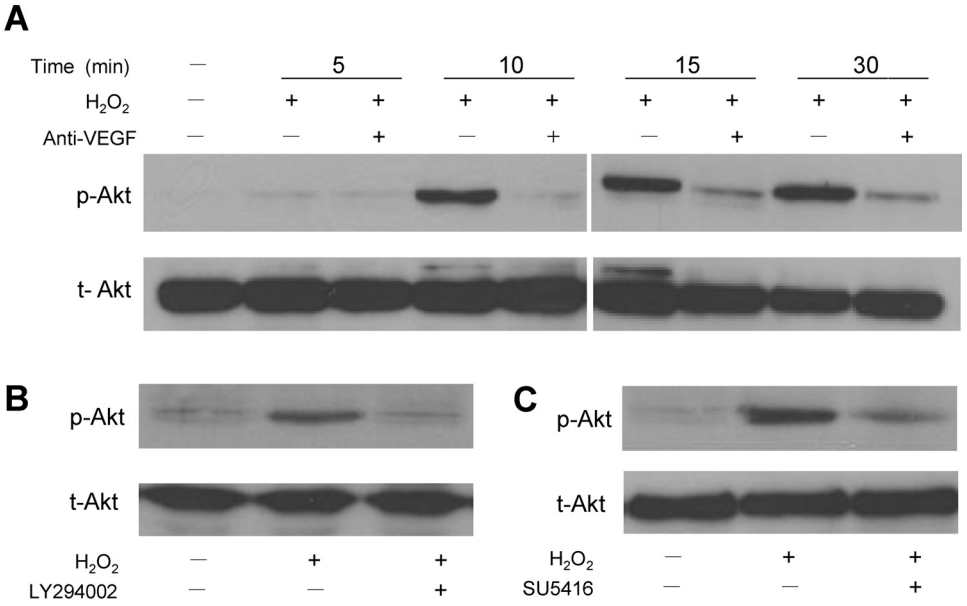

FIGURE 5. Akt phosphorylation by the autocrine VEGF-A and its receptor activation pathway. (**A**) An immunoblot probed with antibody to phosphor-Akt (p-Akt) (Ser473) and antibody to total-Akt (t-Akt), a control for gel loading. ARPE-19 cells were treated with 300 μM H_2O_2 for various times, with or 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.

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 influences 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 has been postulated to compensate for oxidative injury and to prevent apoptotic cell death.[12] Blocking PI3K-Akt significantly enhances H_2O_2-induced RPE cell apoptosis and cell death.[12] 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 pathologic specimens of CNV, RPE cells show excessive proliferation and resultant subretinal scarring.[8] It is not known whether this effect is attributable to loss of RPE cell function under chronic oxidative stress or to 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 disease (e.g., AMD) is present, RPE cells adjacent to CNV undergo transformation and proliferation. Thus, RPE cells under our experimental conditions may simulate those in an in vivo pathologic lesion, compared with 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 disease, but not when the RPE is normal. Another important indication for anti-VEGF-A treatment is diabetic retinopathy, where RPE cells are exposed to a pathologic level of oxidative stress in vivo.

We found that RPE cells secreted not only VEGF-A but also sVEGF-R1, and production of sVEGF-R1 appeared to be regulated by the environmental level of VEGF-A. sVEGF-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] sVEGF-R1 negatively modulates developmental blood vessel formation by inhibition of signaling through VEGF-R2. We found that sVEGF-R1 may 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 sVEGF-R1, the secreted extracellular domain of VEGF-R1.

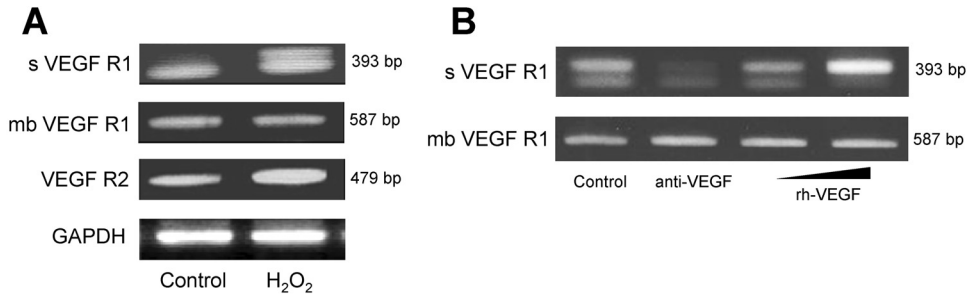

FIGURE 6. Expression of soluble VEGF-R1 by autocrine VEGF signaling under conditions of H_2O_2 stress. Soluble VEGF-R1 (sVEGF-R1) acted as an effective signaling modulator by regulating the availability of free VEGF in the microenvironment with VEGF-R2 then functioning as the primary receptor for VEGF. (**A**) Gene expression of sVEGF-R1, mbVEGF-R1,and VEGF-R2, after 1hour of H_2O_2 stress. (**B**) Gene expression of sVEGF-R1 and mbVEGF-R1 after treatment with anti-VEGF antibody or rhVEGF.

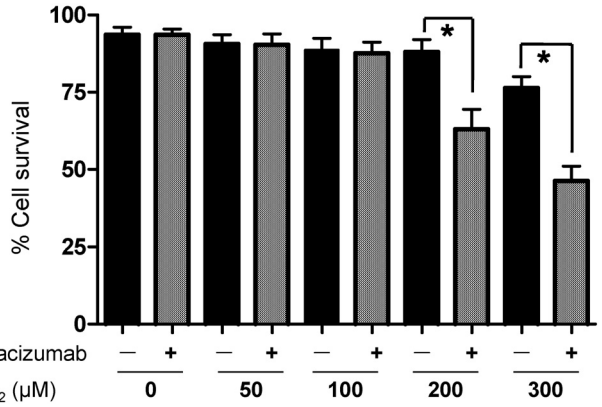

FIGURE 7. Effect of bevacizumab on H_2O_2-induced ARPE-19 cell death. Cell survival analysis by flow cytometry, using annexin V-FITC/DAPI-labeled cells, after pretreatment with bevacizumab (2.5 mg/mL) and H_2O_2 at various concentrations for 16 hours.

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 a different concentration did not cause any functional and morphologic retinal toxicity.[36–38] In vitro cellular assays examining exposure to bevacizumab have shown little toxic effect on ganglion cells, neuroretinal cells, RPE cells, choroidal endothelial cells, and corneal epithelial cells.[39–43] However, in a recent rabbit eye study, the TUNEL method showed that increasing the dosage with intravitreal bevacizumab can cause nuclear DNA fragmentation in the outer retinal layers.[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 (Fig. 7). However, we used a greater dose of bevacizumab than is used 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 needed.

The present study provides evidence that VEGF-A assists in RPE cell survival when cells are exposed to oxidative stress and that 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, influences 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, may affect treatment outcomes (survival of RPE cells, restoration of outer BRB, or geographic atrophy) when anti-VEGF-A treatment is used in patients with neovascular AMD.

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