Sublytic Membrane-Attack-Complex (MAC) Activation Alters Regulated Rather than Constitutive Vascular Endothelial Growth Factor (VEGF) Secretion in Retinal Pigment Epithelium Monolayers*

Kannan Kunchithapautham and Bärbel Rohrer

From the Departments of Ophthalmology and Neurosciences Division of Research, Medical University of South Carolina, Charleston, South Carolina 29425

Uncontrolled activation of the alternative complement pathway and secretion of vascular endothelial growth factor (VEGF) are thought to be associated with age-related macular degeneration (AMD). Previously, we have shown that in RPE monolayers, oxidative-stress reduced complement inhibition on the cell surface. The resulting increased level of sublytic complement activation resulted in VEGF release, which disrupted the barrier function of these cells as determined by transepithelial resistance (TER) measurements. Induced rather than basal VEGF release in RPE is thought to be controlled by different mechanisms, including voltage-dependent calcium channel (VDCC) activation and mitogen-activated protein kinases. Here we examined the potential intracellular links between sublytic complement activation and VEGF release in RPE cells challenged with \( \text{H}_2\text{O}_2 \) and complement-sufficient membrane-attack complex (MAC) activation.

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD),

AGE-RELATED MACULAR DEGENERATION

This work was supported, in whole or in part, by Grant R01 EY019320 from the National Institutes of Health, Grant I01 RX000444 from the Dept. of Veterans Affairs, Foundation Fighting Blindness, and an unrestricted grant to Medical University of South Carolina from Research to Prevent Blindness, Inc.

1 A Research to Prevent Blindness Olga Keith Weiss Scholar. To whom correspondence should be addressed: Department of Ophthalmology, Medical University of South Carolina, 167 Ashley Ave., SEI 511, Charleston, SC 29425. Tel.: 843-792-5086; Fax: 843-792-1723; E-mail: rohrer@musc.edu.

2 The abbreviations used are: AMD, age-related macular degeneration; RPE, retinal pigment epithelium; TER, trans-epithelial resistance; VEGF, vascular endothelial growth factor; MAC, membrane attack complex; VDCC, voltage-dependent calcium channel; CSS, complement-sufficient serum; CFB, complement factor B; CFH, complement factor H; C1INH, complement 1 inhibitor; CNV, choroidal neovascularization.

* This work was supported, in whole or in part, by Grant R01 EY019320 from the National Institutes of Health, Grant I01 RX000444 from the Dept. of Veterans Affairs, Foundation Fighting Blindness, and an unrestricted grant to Medical University of South Carolina from Research to Prevent Blindness, Inc.

Received for publication, December 21, 2010, and in revised form, April 27, 2011 Published, JBC Papers in Press, May 12, 2011, DOI 10.1074/jbc.M110.214593

VOLUME 286 • NUMBER 27
hypothesis that the alternative pathway may play a critical role in driving the pathology of AMD. Thus, the concept emerges that abnormalities in controlling the AP may lead to inflammation at the level of the RPE/Bruch’s membrane, generating a pathological environment that includes drusen formation and VEGF production and secretion, which is favorable for the development of AMD.

To further examine this concept, we investigated RPE cells (ARPE-19 cells) grown as stable monolayers (18). These monolayers exhibit stable transepithelial resistance of \( \sim 40 - 45 \text{ ohm/cm}^2 \); are polarized; and stain for markers of tight and adherence junctions (19). In addition, the Cheetham group has shown that ARPE-19 cells when grown as monolayers, are resistant to oxidative damage; while subconfluent ARPE-19 cells exposed to oxidative stress (\( \geq 0.1 \text{ mM } \text{H}_2\text{O}_2 \)) undergo apoptosis (20), those grown in monolayers show no sign of damage up to 0.5 mM (19). The earliest sign of damage occurs with \( \text{H}_2\text{O}_2 \) concentrations above 1 mM, resulting in a reduction of transepithelial resistance (TER) (19). Thus, we asked whether a concentration that did not affect the epithelial integrity (0.1 mM \( \text{H}_2\text{O}_2 \)), sensitized the cells to complement attack. Based on these experiments (18), we proposed a dual-hit hypothesis in RPE damage, generating a conducive environment for AMD pathology. Oxidative stress in RPE was found to decrease the levels of alternative pathway inhibition by reducing surface expression of the complement inhibitors, CD55 and CD59, and by impairing alternative pathway regulation at the cell surface by factor H present within the serum. Together, these changes sensitized the RPE to complement attack, resulting in increased VEGF secretion and TER reduction.

Here we extend these analyses by investigating how sublytic MAC might be controlling VEGF release and expression. Sublytic MAC-activation results in the formation of discrete channels consisting of a tetramolecular C5b-8 and the tubular lytic MAC-activation results in the formation of discrete channels. The Strauss’ laboratory has shown that VEGF secretion can be controlled by the activity of voltage-gated calcium channels (VDCC), which themselves are regulated by Src kinase (21); whereas work by Klettner and Roide has shown that VEGF secretion from RPE cells is controlled by two pathways (P38 MAP kinase controls constitutive secretion, whereas Erk kinase signaling controls stimulated secretion (22)). The results presented here demonstrate that sublytic MAC utilizes the regulated VEGF secretory pathway to increase VEGF release and production. These results are discussed in the context of VEGF blocking strategies that interfere with both basal VEGF levels required for the health of ocular tissues and the pathological VEGF production seen in ocular inflammatory diseases.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The reagents used in these studies included pooled normal human serum (NHS (Quidel)) as a source of complement proteins. Primary antibodies to phospho-P38, P38; phospho-Erk, Erk; phospho-Src, Src; and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA); the primary antibody to Ras was obtained from Millipore (Billerica, MA). To analyze phosphoproteins, a protease inhibitor mixture was added (Sigma Aldrich). Species-specific secondary antibodies were from Jackson ImmunoResearch (West Grove, PA) and MP Biomedicals, Inc. (Santa Ana, CA). The VEGFR-1/2 inhibitor, SU5416 (Chemicon; now Millipore), was used to block the effects of VEGF. SU5416 (Z-[2-(4-dimethylpyrrolyl)-5-yl]-methylidienyl]-2-indolone) is a lipophilic synthetic receptor tyrosine kinase inhibitor, which inhibits VEGFR-1/2 by binding to the ATP-binding pocket within the kinase domain of the receptor. SU5416 has been shown to inhibit VEGF-dependent endothelial cell proliferation in vitro and in animal models. Nifedipine (TOCRIS Bioscience, Ellisville, MI) is a dihydropyridine or L-type calcium channel blocker. It was used to stabilize the VDCCs in the closed position as described in primary human RPE cells (21). Kinase inhibition was achieved using U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-amino-phenylthio)butadiene; Promega), a highly selective inhibitor of both MEK1 and MEK2 (23); FTS (5-trans, transfarnesylthiosalicylic acid; Cayman Chemical Co., Ann Arbor, MI), a non-toxic but selective Ras antagonist (24); PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; Calbiochem), a potent and reversible, ATP-competitive inhibitor of c-Src (25); as well as SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-1H-imidazol-5-yl]pyridine; Promega), a potent pyridinyl imidazole inhibitor of P38 MAP kinase (26).

**Cell Culture System**—Experiments were performed using ARPE-19 cells, a human retinal pigment epithelial cell line that displays the differentiated phenotype of RPE cells, and forms a polarized monolayer on Transwell filters (Costar; (27, 28)). Cells were grown in DMEM-F12 (Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin:streptomycin as mono-layer growth media. Cells were grown in monolayers show no sign of damage up to 0.5 mM (19). TER measurements, which we have previously shown does not alter survival or monolayer formation in these cells (29). TER of the cell monolayer on the Transwell filters was determined by measuring the resistance across the monolayer with an EVOM volt-ohmmeter (World Precision Instruments) (18). The value for cell monolayers was determined by subtracting the TER for filters without cells and then multiplying by the surface area of the filters. Cell monolayers were considered stable when TER was repeatedly measured as \( \sim 40 - 45 \text{ \Omega/cm}^2 \) (29). TER measurements, which are proportional to membrane permeability, are an accepted readout for the barrier function of an RPE monolayer (27, 29).

As a model of oxidative stress, stable ARPE-19 cell monolayers were treated with 0.5 mM of \( \text{H}_2\text{O}_2 \). It has previously been reported that doses of up to 1 mM are not cytotoxic, and do not lead to disruption of barrier function in these cells (19). After treatment with \( \text{H}_2\text{O}_2 \) monolayers were immediately exposed to 25% NHS as a source of complement proteins (18).

**Quantitative RT-PCR**—ARPE-19 cells from control and experimental sets were collected and stored at 80 °C until used. Quantitative RT-PCR analyses were performed as described in detail previously (30). Primers used were: \( \beta \)-actin, forward: 5’-AAATCTGGCACCACACCTTC-3’ and reverse: 5’-GGG-GTTTGAAGGTCTCAAA-3’; VEGF forward: 5’-AAGGA-
GGAGGCAGAATCAT-3' and reverse: 5'-CACACAGGTGCTTTGAGA-3'; zfp36 forward: 5'-ATCGCCACCCCAA-TACAAG-3' and reverse: 5'-GTCTTGCTAGGTGTTGTGGA-3'. Real-time PCR analyses were performed in triplicate in a GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using standard cycling conditions. Quantitative values were obtained by the cycle number.

ELISA for VEGF—To measure production of VEGF by ARPE-19 cells, apical and basal supernatants were concentrated (Amicon Ultra-4, 3000 Da cutoff; Millipore), solubilized in Celllytic MT (mammalian tissue lysis/extraction reagent; Sigma) and centrifuged at 20,000 × g for 5 min. Microplates were coated with the anti-human VEGF polyclonal capture antibody (Antigenix America, Inc.; Huntington Station, NY) (18), and 100 μl of supernatant was added. The captured proteins were detected with the same VEGF-specific antibody conjugated to horseradish peroxidase (HRP), followed by development with the chromogenic substrate, OPD (Sigma Aldrich). Product development was assayed by measuring absorbance at 492 nm. Aliquots were assayed in duplicate, and values compared with a VEGF dose-response curve.

Western Blot Analysis—Cell extracts were separated by electrophoresis on a 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA), and proteins were transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies; antibody binding was visualized using a chemiluminescence detection kit (Immobilon Western; Millipore Corporation, Billerica, MA). The intensity of the bands was quantified using the Alpha Innotech Fluorchem 9900 imaging system running Alpha Ease FC Software 3.3 (Alpha Innotech, San Leandro, CA). As a loading control, blots were stripped and reprobed with an antibody against GAPDH (Stressgen, Ann Arbor, MI).

Statistics—For data consisting of multiple groups, one-way ANOVA followed by Fisher’s post hoc test (p < 0.05) was used; single comparisons were analyzed by t test analysis (p < 0.05).

RESULTS

Sublytic MAC Activation Leads to Ras, Erk, and Src Activation—Sublytic complement activation, which is known to result in Ca2+ influx through the pores generated by the MAC, is associated with an increase in VEGF secretion (18). Here we examined whether the known kinase pathways involved in VEGF secretion, are activated by sublytic MAC activation. Kinase activation in ARPE-19 cell monolayers was investigated by Western blot analysis at baseline and within a 1-h time course after the exposure of monolayers to 0.5 mM H₂O₂ or 25% NHS for the specified times, cells were collected and analyzed for protein expression by Western blotting. Complement activation rapidly and transiently induced Erk phosphorylation and Ras expression; rapidly and sustainably induced Src-phosphorylation; but partially suppressed P38 phosphorylation. Controls were examined at the 5-min time point, demonstrating that 0.5 mM H₂O₂ or 25% NHS, each administered alone, had no effect on expression levels and phosphorylation status of the signaling molecules.

RESULTS

Sublytic MAC-mediated TER Reduction Is Sensitive to Ras, Erk, and VDCC Inhibition—Sublytic MAC formation is associated with an increase in VEGF secretion, leading to TER reduction, which can be inhibited by using either an alternative pathway inhibitor or by blocking VEGF receptor-1/2 signaling (18). Thus, if the above-investigated pathways were to be involved in VEGF secretion, inhibiting their activation should blunt TER reduction produced by H₂O₂ or NHS (Fig. 2A) and reduce VEGF secretion (Fig. 2B).

As shown previously, TER is stable over the 4-hour recording period in control monolayers, but is reduced by ~50% in those treated with H₂O₂ or NHS (Fig. 2A). Adding 10 μM nifedipine, a specific inhibitor for VDCC (21), to the monolayers exposed to H₂O₂ or NHS almost completely prevented the deterioration of TER. Likewise, the effect of H₂O₂ or NHS is blunted when inhibiting Erk kinase with 10 μM U0126 (23); Ras with 10 μM FTS (24); or Src with 4 μM PP2 (25). Adding the P38 MAP kinase inhibitor, SB203580 at 10 μM (26), had no effect on TER in the presence of H₂O₂ or NHS. None of the inhibitors had any effect on TER in the absence of H₂O₂ or NHS (data not shown).
The effects of the kinase and the VDCC inhibitor on MAC-induced TER reduction were found to be due to their effects on the secretion of VEGF, rather than other nonspecific effects. We have shown previously that co-administering H$_2$O$_2$/H$_1$1001 NHS to either the apical or basal surface resulted in a large fold-increase in VEGF secretion, which was biased toward the apical surface (18). Here we confirmed by using VEGF ELISA assays that VEGF secretion from the RPE monolayers into the apical compartment was induced by H$_2$O$_2$/H$_1$1001 NHS, whereas H$_2$O$_2$ or NHS had no significant effect. While the four inhibitors (nifedipine, U0126, FTS, and PP2) that were found to ameliorate the MAC-induced TER reduction, also significantly blunted the release of VEGF, the P38 MAP kinase inhibitor neither blunted TER reduction induced by H$_2$O$_2$ + NHS nor did it decrease VEGF secretion.

**Sublytic MAC Alters Regulated VEGF Secretion**

The analysis thus far has confirmed that sublytic MAC activation results in VEGF-mediated TER reduction; and that activation can be inhibited by blocking Ras, Erk, Src, and VDCC activity. However, it is unclear whether sublytic MAC triggers these pathways independently, or whether there is any cross-talk. In addition, it needs to be kept in mind that VEGF, which is secreted into the media in response to sublytic MAC-activation, will activate VEGF-R2 receptors that also signal through some of these molecules. To further characterize the intracellular signaling pathways, Western blotting to examine phosphorylation of Erk and Src, and levels of Ras expression were performed (Fig. 3A) and quantified (Fig. 3B) on lysates from cells pretreated with Erk, Src, Ras, VEGF-R2, and VDCC inhibitors, and then exposed to H$_2$O$_2$ + NHS for 5 min. As indicated before, H$_2$O$_2$ + NHS lead to the increase in Erk, Src, and Ras activation. The Erk inhibitor, U0126; the Src inhibitor, PP2; and the Ras inhibitor, FTS, eliminated signaling in their specific pathway. However, PP2 also reduced Ras expression; and FTS slightly reduced Src phosphorylation. In addition, the VEGF-R2 inhibitor, SU5416, blunted Src activation; and the VDCC inhibitor, nifedipine, reduced Src phosphorylation and Ras expression. A summary of expression levels and phosphorylation status of the signaling molecules in the presence of the pathway-specific inhibitors are data expressed as mean ± S.E. (n = 3 per condition).
reduced Src phosphorylation and Ras expression. This analysis places Ras upstream of Erk, but indicates that there is also cross-talk between Src and Ras. Since VEGF-R2 activation was found to stimulate Src phosphorylation, it might therefore amplify secretion of its own ligand. And finally, the effects of nifedipine, resulting in a reduction of Src phosphorylation and Ras expression levels, might be due to the fact that inhibiting the VDCC reduced VEGF secretion may have in part prevented activation of the VEGF-R2-dependent kinase activity.

**Sublytic MAC Activation Increases VEGF Expression via the mRNA-destabilizing Protein Tristetraprolin (zfp36)—VEGF expression is tightly regulated. Hypoxia, in part through the transcription factor Hif1α, is thought to be the principal stimulus for VEGF secretion from the RPE (31); and immortalized human RPE cells have been shown to secrete VEGF in response to oxidative stress, with higher secretion toward the retina side (32). A second level of control is at the level of cytoplasmic resistance; effects that could be prevented or significantly blunted by tristetraprolin or zfp36; and loss of zfp36 can result in increased VEGF stability and hence protein production (36). Here, we asked whether sublytic MAC activation affects VEGF and zfp36 mRNA levels, and whether those levels are affected by the intracellular signaling pathways mediated by H2O2 or NHS. VEGF and zfp36 mRNA levels were measured over the first 4 h of exposure of RPE monolayers to H2O2 + NHS, or either H2O2 or NHS alone (Fig. 4A). Zfp36 mRNA levels increased rapidly after exposure to H2O2 + NHS, while VEGF levels were unaffected. During the 4 h of exposure time an inverse correlation was identified between zfp36 and VEGF. When the levels of the destabilizing protein zfp36 dropped, levels of VEGF mRNA increased. Exposure of monolayers with H2O2 or NHS alone for 4 h had no effect on the balance between zfp36 and VEGF mRNA levels; zfp36 mRNA levels remained elevated, while VEGF levels remained at baseline. As predicted based on the VEGF secretion data, cells exposed to H2O2 + NHS that were pretreated with Erk, Src, Ras, VEGF-R2, and VDCC inhibitors showed little increase in VEGF mRNA expression. While the levels of zfp36 mRNA levels were reduced in the inhibitor-treated when compared with cells exposed to H2O2 + NHS, levels were increased significantly by ~4-fold when compared with untreated control cells (Fig. 4B). Collectively, these data suggest that zfp36 expression is associated with a reduction in VEGF expression, which in turn can alter the pathological phenotype of the RPE. However, additional experiments investigating zfp36 protein levels, phosphorylation status and mRNA and protein stability are needed to further characterize this interaction.

**DISCUSSION**

Our results demonstrate that sublytic MAC utilizes the regulated VEGF secretory pathway to increase VEGF release. Sublytic MAC was found to stimulate intracellular signaling molecules known to be part of the regulated secretory pathway such as Ras, Erk and Src, but not the basal VEGF secretory pathway, which is mediated by activation of P38 MAP kinase. Activity in the Erk/Ras and Src/VDCC pathways led to an increase in VEGF secretion followed by an impairment of transepithelial resistance; effects that could be prevented or significantly blunted by their respective inhibitors. Finally, activity in these pathways was correlated with a decrease in VEGF mRNA expression and a concomitant increase in the VEGF mRNA destabilizing protein, zfp36.

The complement system is an essential part of the evolutionarily ancient innate immune system, involved in eliminating foreign antigens and pathogens (reviewed in Ref. 37, 38). However, inappropriate or excessive complement activation is also involved in the pathogenesis of autoimmune, inflammatory, and ischemic disease states (reviewed in Ref. 39). The complement system can be activated by three distinct pathways: the classical (CP), lectin (LP), and alternative pathway (AP) (40). All three pathways lead to the generation of a C3 convertase enzyme complex. The convertases cleave C3 to produce C3a and a C3b fragment, and participate in the formation of C5 convertase, which cleaves C5 to yield C5b and the soluble anaphylatoxin, C5a. Formation of C5b initiates the terminal com-

**FIGURE 4. Complement activation on ARPE-19 cells induces the expression of VEGF mRNA and reduces expression of zfp36 mRNA, a known destabilizer of VEGF transcripts.** Serum-deprived ARPE-19 cell monolayers with stable TER were treated with 0.5 mM H2O2 and 25% NHS in the presence and absence of pathway-specific inhibitors, and cells were collected after 4 h, or at the time points specified. VEGF and zfp mRNA levels were determined by QRT-PCR. A, exposure of monolayers to oxidative stress or serum resulted in an increase in zfp36, without affecting VEGF mRNA levels (3 h of exposure. Complement activation (H2O2 and NHS) however, led to a rapid increase in VEGF mRNA expression, and a concomitant decrease in zfp. B, pretreatment of cells exposed to H2O2 + NHS with Erk, Src, Ras, VEGF-R2, and VDCC inhibitors significantly blunted the increase in VEGF mRNA expression, and concomitantly increased zfp36 mRNA levels. On the other hand, cells pretreated with the P38 inhibitor had VEGF and zfp36 expression levels comparable to the H2O2 + NHS-treated controls. Data are expressed as mean ± S.E. (n = 3 per condition).
Sublytic MAC Alters Regulated VEGF Secretion

Sublytic pathway resulting in the sequential assembly of complement proteins, C6, C7, C8, and C9, to form the cytolytic membrane attack complex (MAC or C5b-9). Triggering of this final pathway can lead to either lytic or sublytic activation. Lytic activation results in the assembly of the membrane attack complex, which is the cytolytic end-product of the complement cascade; it forms a transmembrane channel, causing osmotic lysis of the target cell. Sublytic activation occurs when the number of channels assembled on the cell surface is limited, which does not lead to the destruction of the target cell. At sublytic doses, the complement MAC complex has a wide range of effects on many cell types leading to changes in cellular responses such as secretion, adherence, aggregation, chemotaxis, cell division, or membrane function (reviewed in Ref. 41; examples in Refs. 42–45). Sublytic complement also induces increased cell resistance to lytic doses of complement. The transmembrane channel consists of the tetramolecular C5b-8 and tubular poly-C9 complex. The diameter of the pore created varies according to the amount of C9 molecules available to complex with bound C5b-8. Binding of the first molecule of C9 initiates C9 oligomerization at the site of MAC assembly; and once at least 12 molecules are incorporated into the complex, a discrete channel structure is formed (46). Human serum contains 42–77 μg/ml C9, which is 10–20-fold more than what is required for sublytic MAC activation; Sala-Sala-Newby et al. (47) have shown in a cell-based system using HeLa cells, that the threshold for sublytic MAC activation lies at ~4 μg/ml of C9. The pore allows free movement of molecules in and out of the cell, since it has a hydrophilic internal face that allows the passage of water. Important for this work, Ca2+ influx has been shown to be one of the general consequences of MAC activation (48), resulting in Ca2+ oscillations that have been shown to last up to 45 min, in oligodendrocytes (49). In muscle cells, Jackson et al. (50) have shown using patch-clamp analysis of individual MAC channels, that these pores rapidly change between conducting and non-conducting states. Sublytic MAC activation is transient, exhibiting a half-life of ~40 min, with MAC being removed from the cell surface by either outward or inward vesiculation (51). This process is Ca2+-calmodulin dependent (52). Finally, there are a number of different inhibitors that prevent MAC assembly. The membrane-bound, GPI-anchored inhibitor, CD59, acts by binding to the C8 and the C9 complement proteins, and thereby prevents formation of the lytic pore (53). In addition, the soluble inhibitors, S-protein (vitronectin) (54) or clusterin (complement-associated protein SP-40,40; complement cytolysis inhibitor; aging-associated gene 4 protein; Ku70-binding protein 1, NA1/NA2; testosterone-repressed prostate message 2; or apolipoprotein J) (55), which bind to the C3b-7 structure, prevent its attachment to cell membranes by rendering it water-soluble and lytic inactive. Thus, in ARPE-19 cells, due to the presence of complement inhibitors CD46, 55, and 59 on the cell surface, complement activation leads to transient MAC activation (18). The transient insertion of these transmembrane channels is expected to lead to Ca2+ influx, resulting in membrane depolarization and subsequent changes in VEGF secretion. Unfortunately, the calcium requirement could not be tested directly. Although the alternative pathway is active in the presence of Mg2+-EGTA, concentrations sufficient to eliminate Ca2+ in media containing 25% serum, significantly impaired TER. We expect to answer this question in future studies using calcium imaging, as well as patch clamp recordings to examine membrane potential and ion channel activity.

Overall, the data suggest the following possible mechanisms of VEGF secretion (Fig. 5). VEGF is typically present in granular vesicles (56) and exhibits polarized secretion in RPE cells (e.g. Ref. 29, 32) via a saturable process (57). Regulated secretion of polypeptides present in granular vesicles occurs via exocytosis triggered by Ca2+. Sublytic complement activation, which is due to the transient insertion of MAC, generating pores that allow the influx of ions, in particular Ca2+ and Na+, depolarizes the cell membrane. Membrane depolarization and the local influx of Ca2+ could therefore trigger the following consecutive or parallel events to result in the observed VEGF secretion. First, membrane depolarization is known to activate voltage-dependent calcium channels (VDCC), resulting in the local influx of Ca2+ required for VEGF release. This mechanism can be amplified by the MAC-mediated influx of Ca2+ leading to Src activation, which activates the VDCC. Interestingly, RPE cells from AMD patients show increased VEGF release via this Src/VDCC pathway (21). Second, Ca2+ influx is also known to activate the Ras/Erk pathway (58–60), a pathway that has been shown to be involved in the regulated secretion of VEGF (22). Because Erk activity has been shown to result in a transient rise in intracellular Ca2+ (61), this might be the source of Ca2+ required to secrete VEGF via the Ras/Erk pathway. Thus, there seems to be at least two intracellular signaling pathways that are triggered by sublytic MAC formation, Src/VDCC and Ras/Erk. Based on the transient nature of the increase in Erk phosphorylation and Ras expression levels, and the sustained increase in...
Ssrc phosphorylation, the two mechanisms together might be responsible for an initial burst followed by a more sustained level of VEGF secretion. Activity in these two pathways might mediate the VEGF-induced reduction in TER, which plateaus within 1–4 h after stimulation (18, 29). The inhibitor experiments presented in Fig. 3 suggest that there is crosstalk between these two pathways at the level of Src and Ras. Src-mediated signaling via Ras has been reported in other systems (62, 63); however, signaling in the other direction appears to be less common. In summary, sublytic MAC-mediated VEGF secretion occurs via two known pathways that control regulated, rather than basal VEGF release.

As outlined above, the expression of angiogenic factors such as VEGF is carefully regulated, involving the tightly balanced transcription factor, Hif1α (31), and a mechanism that controls its mRNA stability through the mRNA destabilizing protein, zfp36 (36). Here, we reported on two observations. First, exposing serum-deprived RPE monolayers (serum was withdrawn in all experiments 5–7 days prior to the experiment to allow for complement activation with 25% serum) to H2O2, NHS, or H2O2 + NHS resulted in a rapid increase in zfp36 without affecting VEGF mRNA levels. Zfp36 has been shown to be induced by a variety of conditions, including growth factor present in serum (64) and oxidative stress (65). Second, we showed that sublytic MAC-activation had opposing effects on VEGF and zfp36 mRNA levels; H2O2 + NHS stimulation increased VEGF and decreased zfp36 mRNA levels, whereas the inhibitors to those pathways involved in stimulating VEGF secretion, had the opposite effects. It has been reported that Erk activity is associated with a decrease in zfp36 activity (36, 66), linking Erk activity with VEGF mRNA expression. In addition, it has been shown that both Src and Ras (66) can activate VEGF expression, presumably through the phosphorylation of nuclear transcription factors; and since phosphorylated zfp36 is less active, this may be an alternative means to regulate VEGF levels in the MAC-stimulated RPE cells. However, additional experiments investigating zfp36 protein levels, phosphorylation status, and mRNA and protein stability are needed to further distinguish between these possibilities.

VEGF is an important signaling protein involved in both de novo formation of the embryonic circulatory system and angiogenesis, and signals via two tyrosine kinase transmembrane VEGF receptors, flt-1 and flk-1 (67). The RPE is a major source of VEGF (e.g. Ref. 68). Histopathological studies of CNV membranes from patients with AMD have demonstrated the presence of VEGF and its receptors (e.g. Ref. 69). Animal studies support a role for increased VEGF of RPE origin in CNV progression (70). Given the importance of VEGF, currently therapeutic approaches targeting the regulated VEGF secretory pathway to reduce VEGF release and production should be investigated; and since the alternative pathway of complement controls the regulated VEGF secretory pathway, interfering at the level of complement to reduce inflammation and pathological VEGF secretion (e.g. Ref. 73) might provide a unique strategy for the treatment of AMD.

Acknowledgments—We thank Joshua Thurman (University of Colorado Denver School of Medicine, Denver, CO) for helpful discussions and Luanna Bartholomew for critical review (Medical University of South Carolina, Charleston, SC). Animal studies were conducted in a facility constructed with support from the National Institutes of Health (C06 RR015455).

REFERENCES

1. Hageman, G. S., Luthert, P. J., Victor Chong, N. H., Johnson, L. V., Anderson, D. H., and Mullins, R. F. (2001) Prog. Retin. Eye Res. 20, 705–732
2. Tomany, S. C., Wang, J. I., Van Leeuwen, R., Klein, R., Mitchell, P., Vingerburn, M. K., and Ablonczy, Z., Tomlinson, S., Holers, V. M., and Rohrer, B. (2008) Am. J. Ophthalmol. 145, 176–182
3. Anderson, D. H., Mullins, R. F., Hageman, G. S., and Johnson, L. V. (2002) Am. J. Pathol. 160, 852–864
4. Chong, N. H., Keonin, J., Luthert, P. J., Frennesson, C. I., Weingeist, D. M., Collins, A., Stone, E., and Lotery, A. (2008) Ocul. Immunol. Inflamm. 16, 175–182
5. Anderson, D. H., Mullins, R. F., Hageman, G. S., and Johnson, L. V. (2002) Am. J. Ophthalmol. 134, 411–431
6. Chong, N. H., Keonin, J., Luthert, P. J., Frennesson, C. I., Wingeist, D. M., Wolf, R. L., Mullins, R. F., and Hageman, G. S. (2005) Am. J. Pathol. 166, 241–251
7. Johnson, L. V., Leitner, W. P., Staples, M. K., and Anderson, D. H. (2001) Invest. Ophthalmol. Vis. Sci. 42, 1564–1571
8. Park, K. H., Ryu, E., Tosakulwong, N., Wu, Y., and Edwards, A. O. (2009) Mol. Vis. 15, 200–207
9. Zerib, J., Richard, F., Puche, N., Leveziel, N., Cohen, S. Y., Korbelenik, J.-F., Sahel, J., Munnich, A., Kaplan, J., Rozet, J. M., and Souied, E. H. (2010) Mol. Vis. 16, 1324–1330
10. Schmidt, S., Hauser, M. A., Scott, W. K., Postel, E. A., Agarwal, A., Gallins, P., Wong, F., Chen, Y. S., Spencer, K., Schnetz-Boutaud, N., Haines, J. L., and Luanna Bartholomew for critical review (Medical University of South Carolina, Charleston, SC). Animal studies were conducted in a facility constructed with support from the National Institutes of Health (C06 RR015455).
Sublytic MAC Alters Regulated VEGF Secretion

20. Choudhary, S., Xiao, T., Srivastava, S., Zhang, W., Chan, L. L., Vergara, L. A., Van Kuijk, F. J., and Ansari, N. H. (2005) Toxicol. Appl. Pharmacol. 204, 122–134
21. Rosenthal, R., Heimann, H., Agostini, H., Martin, G., Hansen, L. L., and Strauss, O. (2007) Mol. Vis. 13, 443–456
22. Klettner, A., and Roeder, J. (2009) Graefes Arch. Clin. Exp. Ophthalmol. 247, 1487–1492
23. Samuel, W., Kutty, R. K., Sekhar, S., Vijayasarthay, C., Wiggert, B., and Redmond, T. M. (2008) J. Neurochem. 106, 591–602
24. Schneider-Mercck, T., Borbath, I., Charette, N., De Saeger, C., Abarca, J., Leclercq, I., Horsmans, Y., and Stéphane, P. (2009) Eur. J. Cancer 45, 2050–2060
25. Finnegan, S., Mackey, A. M., and Cotter, T. G. (2010) Eur. J. Neurosci. 32, 322–334
26. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L., and Lee, J. C. (1997) J. Biol. Chem. 272, 12116–12121
27. Dunn, K. C., Aotaki-Keen, A. E., Putkey, F. R., and Hjelmeland, L. M. (1996) Exp. Eye Res. 62, 155–169
28. Dunn, K. C., Marmostein, A. D., Bonilha, V. L., Rodriguez-Boulan, E., Giordano, F., and Hjelmeland, L. M. (1998) Invest. Ophthalmol. Vis. Sci. 39, 2744–2749
29. Ablonzcy, Z., and Crosson, C. E. (2007) Exp. Eye Res. 85, 762–771
30. Lohr, H. R., Kunchitaphaathum, K., Sharma, A. K., and Rohrer, B. (2006) Exp. Eye Res. 83, 380–389
31. Pouyssegur, J., Dayan, F., and Mazure, N. M. (2006) Nature 441, 437–443
32. Kannan, R., Zhang, N., Sreekumar, P. G., Spee, C. K., Rodriguez, A., Barron, E., and Hinton, D. R. (2006) Mol. Vis. 12, 1649–1659
33. Chen, C. Y., and Shyu, A. B. (1995) Trends Biochem. Sci. 20, 465–470
34. Guhaniyogi, J., and Brewer, G. (2001) Gene 265, 11–23
35. Barreau, C., Paillard, L., and Osborne, H. B. (2005) Nucleic Acids Res. 33, 7138–7150
36. Brennan, S. E., Kuwano, Y., Alkharouf, N., Blackshear, P. J., Gorospe, M., and Wilson, G. M. (2009) Cancer Res. 69, 5168–5176
37. Fearon, D. T. (1998) Physiol. Genomics 3, 253–265
38. Stephans, C. L., and Henkart, P. A. (1979) J. Immunol. 122, 455–458
39. Lai, W. S., Thompson, M. J., and Blackshear, P. J. (1998) J. Biol. Chem. 273, 506–517
40. Edwards, M. G., Sarkar, D., Klopp, R., Morrow, J. D., Weindruch, R., and Prolla, T. A. (2003) Physiol. Genomics 13, 119–127
41. Essafi-Benkhadir, K., Onesto, C., Moroni, C., and Pages, G. (2007) Mol. Biol. Cell 18, 4648–4658
42. Rusanescu, G., and D’Amore, P. A. (1996) Cytokine Growth Factor Rev. 7, 259–270
43. Marmorstine, A. D., Csaky, K. G., Baffi, J., Lam, L., Rahaal, F., and Rodriguez-Boulan, E. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 3248–3253
44. Yun, H. Y., Dawson, V. L., and Dawson, T. M. (1999) Diabetes Res. Clin. Pract. 45, 113–115
45. Rosen, L. B., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994) Neuron 12, 1207–1211
46. Lohr, H., Kunchitaphaathum, K., Sharma, A. K., and Rohrer, B. (2006) Exp. Eye Res. 83, 380–389