Sublytic membrane-attack-complex (MAC) activation alters regulated rather than constitutive VEGF secretion in retinal pigment epithelium monolayers

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Running head: Sublytic MAC alters regulated VEGF secretion

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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 facility 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 H$_2$O$_2$ and complement-sufficient normal human serum (NHS). Disruption of barrier function by H$_2$O$_2$ + NHS rapidly increased Ras expression and Erk and Src phosphorylation, but had no effect on P38 phosphorylation. Either treatment alone had little effect. TER reduction could be attenuated by inhibiting Ras, Erk and Src activation, or blocking VDCC or VEGF-R2 activation, but not by inhibiting P38. Combinatorial analysis of inhibitor effects demonstrated that sublytic complement activation triggers VEGF secretion via two pathways, Src and Ras-Erk, with the latter being amplified by VEGF-R2 activation, but has no effect on constitutive VEGF secretion mediated via P38. Finally, effects on TER were directly correlated with release of VEGF; and sublytic MAC activation decreased levels of zfp36, a negative modulator of VEGF transcription, resulting in increased VEGF expression. Taken together, identifying how sublytic MAC induces VEGF expression and secretion might offer opportunities to selectively inhibit pathological VEGF release only.

Age-related macular degeneration (AMD), the leading cause of blindness for Americans over age sixty, is a slowly progressing multifactorial disease involving genetic abnormalities and environmental insults. While mechanistic studies have shown that inflammation (1) and smoking (2) are fundamental components of AMD, genetic studies have demonstrated that polymorphisms in different complement proteins each increase the risk for developing AMD. Genetic variations with a number of complement components, including those of required activators (complement factor B, CFB) (3), inhibitors (complement factor H, CFH (4-6); complement 1 inhibitor, C1INH, although the evidence is conflicting (7,8)) and essential components in the complement cascade (complement factor 2 and 3, C2 (3) and C3 (9)) have been found to be associated with all forms of AMD. In particular, one of the most detrimental mutations occurs in CFH (fH risk haplotype). The only environmental agent unequivocally linked to AMD is nicotine (10), presumably by generating oxidative stress. Overall, oxidative stress is thought to be an important risk factor, presumably impacting RPE function (11). Current available treatments for AMD focus on the late stage of the disease (choroidal neovascularization; CNV). To this end, therapies have focused on either physically or pharmacologically interrupting blood vessel growth (summarized in the following review article (12)), in particular, by using anti-angiogenic drugs (VEGF-inhibitors) that deplete the new blood vessels of an essential growth factor. Finally, what all stages of AMD have in common is varying degrees of damage to the RPE/Bruch’s membrane, which ulti-
mately results in damage to the underlying photoreceptors.

Early AMD is characterized by the formation of drusen at the choroid/RPE interface (1,13-15). Drusen contain large numbers of proteins including amyloid proteins, coagulation factors and complement components. A high concentration of complement regulatory proteins and membrane attack complex (MAC), in the area of Bruch’s membrane and the RPE has been documented (1,15-17). MAC deposition density is correlated with severity of AMD and is most pronounced in the macula. Furthermore, there is a correlation between the amount of MAC deposition and the loss of RPE cells (1). These data, combined with the relative lack of immunoglobulins in drusen (which would activate the classical pathway) support the 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 ~40-45 ohm/cm²; 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 (≥0.1 mM H₂O₂) 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 H₂O₂ 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 H₂O₂), 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 poly-C9 complex, which allows for the influx of mainly Na⁺ and Ca²⁺ ions. Local influx of Ca²⁺ might directly control VEGF secretion. However, other pathways should be considered. The Strauss’ lab 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 Roider 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; phosphor-Erk, Erk; phosphor-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 cocktail was added (Sigma Aldrich, St. Louis, MO). 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-3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-2-indolinone) 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, Madison WI), a highly selective inhibitor of both MEK1 and MEK2 (23); FTS (S-trans, transfarnesylthiosalicylic acid; Cayman Chemical Company, 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-methylsulfonylphenyl)-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 (Gibco) with 10% fetal bovine serum (FBS) and 1 x penicillin:streptomycin as monolayers on Transwell filters. FBS was removed completely for the final 5-7 days (2-3 media changes) prior to 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 voltohmometer (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 ~40-45 Ω/cm² (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 H₂O₂. 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 H₂O₂, 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: ß-actin, forward: 5′-AAAATCTGGCACCACACCTTC-3′ and reverse: 5′-GGGGTGTTGAAGGCTCAAAA-3′; VEGF forward: 5′-AAGGAGGAGGCAG AAT- CAT-3′ and reverse: 5′-CACACAGATGCG TTGAAGA-3′; zfp36 forward: 5′-ATCGCC ACCAAAAATACAAG -3′ and reverse: 5′- GTCTTCGCTAGGGTTGGGA-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 20,000g 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 to 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 Ca\(^2+\) 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-hour time course after the exposure of monolayers to 0.5 mM H\(_2\)O\(_2\) + 25% complement-sufficient normal human serum (NHS) (Fig. 1A). In particular we focused on P38 MAP kinase, which participates in constitutive VEGF secretion (22); Erk kinase, which participates in regulated VEGF secretion (22); Src, which increases VDCC-mediated VEGF secretion (21); and finally Ras, which provides a link between Src and Erk signaling.

P38-phosphorylation was found to be reduced by approximately 40% after the exposure of monolayers to H\(_2\)O\(_2\) + NHS, when compared to the overall amount of P38 present in the monolayers (Fig. 1A, 1C), and remained depressed during the time course analyzed (1 hour). A transient increase in phospho-Erk could be detected rapidly within minutes, as could overall Ras levels (Fig. 1A, 1C). Both Erk and Ras activation peaked within 5 minutes after the onset of stimulation (earliest time point analyzed) and returned to baseline levels by 10 (Ras) or 20 minutes (Erk) (Fig. 1A, 1C). In contrast, while the increase in phosphorylation of Src is equally rapid, it remained elevated over the 1-hour investigation period (Fig. 1A, 1C). H\(_2\)O\(_2\) or NHS when administered singly did not lead to any significant alterations in kinase activity (Fig. 1B).

Sublytic MAC-mediated TER reduction is sensitive to Ras, Erk, Src 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 H\(_2\)O\(_2\) + 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 H\(_2\)O\(_2\) + NHS (Fig. 2A). Adding 10 μM nifedipine, a specific inhibitor for VDCC (21), to the monolayers exposed to H\(_2\)O\(_2\) + NHS almost completely prevented the deterioration of TER. Likewise, the effect of H\(_2\)O\(_2\) + NHS is blunted when inhibiting Erk kinase with 10 μM of U0126 (23); Ras with 10 μM FTS (24); or Src with 4 pM of PP2 (25). Adding the P38 MAP kinase inhibitor, SB203580 at 10 μM (26), had no effect on TER in the presence of H\(_2\)O\(_2\) + NHS. None of the inhibitors had any effect on TER in the absence of H\(_2\)O\(_2\) + 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 non-specific effects (Fig. 2B). We have shown previously that co-administering H\(_2\)O\(_2\) + 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\(_2\) + 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-mediated intracellular cross-talk. 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 minutes. As indicated before, H$_2$O$_2$ + NHS lead to the increase in Erk, Src and Ras activation. The Erk inhibitor, U0126, only eliminated Erk activation; the Src inhibitor, PP2, eliminated Src activation and also reduced Ras levels; the Ras inhibitor, FTS, eliminated the increase in Ras expression, completely eliminated Erk, and slightly reduced Src phosphorylation; inhibiting VEGF-R2 with SU5416 blunted Src phosphorylation; and finally the VDCC inhibitor, nifedipine, 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 stability of the mRNA encoding for VEGF. Stability of many cytokines, including VEGF, is controlled by AU-rich elements (ARE), a family of RNA sequences located within the 3′-untranslated region (3′-UTR) (33,34). Specific binding proteins that bind to the ARE accelerate or slow-down the decay of the respective transcript (35). VEGF mRNA has been shown to be destabilized 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 activated by H$_2$O$_2$ + NHS. VEGF and zfp36 mRNA levels were measured over the first four-hours of exposure of RPE monolayers to H$_2$O$_2$ + NHS, or either H$_2$O$_2$ or NHS alone (Fig. 4A). Zfp36 mRNA levels increased rapidly after exposure to H$_2$O$_2$ + NHS, while VEGF levels were unaffected. During the four-hours 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 H$_2$O$_2$ or NHS alone for four hours 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 H$_2$O$_2$ + 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 to cells exposed to H$_2$O$_2$ + NHS, levels were increased significantly by ~4-fold when compared to 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 (37,38)). However, inappropriate or excessive complement activation is also involved in the pathogenesis of auto-immune, inflammatory and ischemic disease states (reviewed in (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 complement 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 (41); examples in (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-Newby and coworkers have shown in a cell-based system using HeLa cells, that the threshold for sublytic MAC activation lies at ~4 µg/mL of C9 (47). 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, Ca\textsuperscript{2+} influx has been shown to be one of the general consequences of MAC activation (48), resulting in Ca\textsuperscript{2+} oscillations that have been shown to last up to 45 minutes, in oligodendrocytes (49). In muscle cells, Jackson and coworkers have shown using patch-clamp analysis of individual MAC channels, that these pores rapidly change between conducting and non-conducting states (50). Sublytic MAC activation is transient, exhibiting a half-life of ~40 minutes, with MAC being removed from the cell surface by either outward or inward vesiculation (51). This process is Ca\textsuperscript{2+}-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 C5b-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 Ca\textsuperscript{2+} 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 Mg\textsuperscript{2+} EGTA, concentrations sufficient to eliminate Ca\textsuperscript{2+} 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., (29,32)) via a saturable process (57). Regulated secretion of polypeptides present in granular vesicles occurs via exocytosis triggered by Ca\textsuperscript{2+}.
Sublytic complement activation, which is due to the transient insertion of MAC, generating pores that allow the influx of ions, in particular Ca\(^{2+}\) and Na\(^{+}\), depolarizes the cell membrane. Membrane depolarization and the local influx of Ca\(^{2+}\) 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 Ca\(^{2+}\) required for VEGF release. This mechanism can be amplified by the MAC-mediated influx of Ca\(^{2+}\), 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, Ca\(^{2+}\) 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). Since Erk activity has been shown to result in a transient rise in intracellular Ca\(^{2+}\) (61), this might be the source of Ca\(^{2+}\) 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 Src 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 hours after stimulation (18,29). The inhibitor experiments presented in Figure 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 H\(_2\)O\(_2\), NHS, or H\(_2\)O\(_2\) + 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; H\(_2\)O\(_2\) + 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., (68)). Histopathological studies of CNV membranes from patients with AMD have demonstrated the presence of VEGF and its receptors (e.g., (69)). Animal studies support a role for increased VEGF of RPE origin in CNV progression (70). Given the importance of VEGF, currently approved therapies are directed at CNV which occurs in patients with wet AMD. Current treatments (Macugen and Lucentis), directed at inhibiting VEGF, have been shown to improve vision and/or slow progression of the disease (71). However, since basal levels of VEGF may be required for maintenance of the adult choroidal vasculature, and VEGF is a critical survival factor in the retina (72), long-term anti-VEGF therapy may not be safe. Thus, focusing on a treatment that reduces the pathologic, rather than the basal VEGF expression may be essential for maintaining normal blood vessel health. Hence, 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., (73)) might provide a unique strategy for the treatment of AMD.

REFERENCES


**FOOTNOTES**

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

**FIGURE LEGENDS**

**Fig. 1.** Complement activation on ARPE-19 cells induces intracellular signaling associated with regulated VEGF secretion. ARPE-19 cells were grown as a monolayer until a stable TER was obtained,
at which point serum was withdrawn for 5-7 days prior to the experiment. A. Following apical treatment with 0.5 mM H$_2$O$_2$ and 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. B. Controls were examined at the 5-minute time point, demonstrating that 0.5 mM H$_2$O$_2$ or 25% NHS, each administered alone, had no effect on expression levels and phosphorylation status of the signaling molecules. C. Summary of expression levels and phosphorylation status of the signaling molecules. Data are expressed as mean ± SEM (n = 3 per condition).

**Fig. 2.** Disruption of barrier function by complement activation on ARPE-19 cells is correlated with activity in pathways known to control regulated secretion of VEGF. Serum-deprived ARPE-19 cell monolayers with stable TER were treated with 0.5 mM H$_2$O$_2$ and 25% NHS in the presence and absence of pathway-specific inhibitors. A. Treatment with H$_2$O$_2$ and serum caused a reduction in transepithelial resistance (TER) by ~40% after 4 hr. Co-administration of nifedipine (a dihydropyridine or L-type calcium channel blocker); U0126 (a highly selective inhibitor of Erk); FTS (a selective Ras antagonist); or PP2 (a reversible, ATP-competitive inhibitor of c-Src) blunted the deterioration of TER induced by H$_2$O$_2$ + 25% NHS; whereas SB203580 (a potent inhibitor of P38 MAP kinase) had no effect. B. After TER measurements, apical supernatants were removed and analyzed for VEGF content. VEGF secretion is not altered by H$_2$O$_2$ or NHS, whereas H$_2$O$_2$ + NHS together resulted in a significant increase in VEGF release. The inhibitors that blunted TER reduction also resulted in a reduction in VEGF secretion. C. When VEGF secretion was plotted against the percentage reduction in TER, a perfect correlation was revealed. Data are expressed as mean ± SEM (n = 3 per condition).

**Fig. 3.** Analysis of cross-talk between signaling pathways induced by complement activation in ARPE-19 cells. ARPE-19 cell monolayers were grown as described in Figure 1. Erk and Src phosphorylation as well as Ras expression were analyzed in the presence of pathway-specific inhibitors. A. Following apical treatment with 0.5 mM H$_2$O$_2$ and 25% NHS for 5 minutes, cells were collected and analyzed for protein expression by Western blotting. Complement activation rapidly induced Erk- and Src-phosphorylation as well as Ras expression. 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. B. Summary of expression levels and phosphorylation status of the signaling molecules in the presence of the pathway-specific inhibitors. Data are expressed as mean ± SEM (n = 3 per condition).

**Fig. 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 H$_2$O$_2$ and 25% NHS in the presence and absence of pathway-specific inhibitors, and cells were collected after 4 hours, 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 hours of exposure. Complement activation (H$_2$O$_2$ and NHS) however, led to a rapid increase in VEGF mRNA expression, and a concomitant decrease in zfp. B. Pretreatment of cells exposed to H$_2$O$_2$ + 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 H$_2$O$_2$ + NHS-treated controls. Data are expressed as mean ± SEM (n = 3 per condition).

**Fig. 5.** Sublytic MAC activation in RPE-cells increases regulated VEGF secretion. VEGF, which is present in granular vesicles, is secreted via exocytosis triggered by Ca$^{2+}$. Sublytic complement activation, results in the influx of Ca$^{2+}$ and Na$^+$ ions, which triggers both Ca$^{2+}$-activated pathways and also de-
polarizes the cell membrane. Membrane depolarization activates voltage-dependent calcium channels (VDCC), allowing for the local influx of Ca\(^{2+}\) required for exocytosis. VDCC activation can be amplified by MAC-mediated influx of Ca\(^{2+}\) leading to Src activation. Ca\(^{2+}\) influx also activates the Ras/Erk pathway known to be involved in the regulated secretion of VEGF. Thus, sublytic MAC formation engages at least two intracellular signaling pathways that result in regulated VEGF secretion: Src/VDCC and Ras/Erk. The inhibitor experiments also revealed crosstalk between these two pathways. Finally, both Ras- and Erk-mediated signaling have been reported to inhibit zfp, which in turn results in increased VEGF expression. Thus, we hypothesize that sublytic MAC-activation engages pathways involved in regulated VEGF secretion rather than basal VEGF release.
Figure 1
Figure 2

(A) [Bar chart with different conditions and their effects on transpulmonary resistance.]

(B) [Bar chart showing VEGF concentration across different conditions.]

(C) [Graph showing a linear relationship between VEGF concentration and % transpulmonary resistance, with a correlation coefficient of R=0.992, P<0.0001.]
Figure 4

(A) Graph showing the fold difference over unrelated control for VEGF and Zfp36 over time (in hours) of H2O2 + NHS.

(B) Graph showing the fold difference over unrelated control for different treatments: H2O2 + NHS, + Erk inhibitor, + Sc inhibitors, + Ras inhibitor, + P38 inhibitor, + VEGF-R2 inhibitor, + VDCC inhibitor.
Figure 5
Sublytic membrane-attack-complex (MAC) activation alters regulated rather than constitutive VEGF secretion in retinal pigment epithelium monolayers
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