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REVIEW ARTICLE

Autocrine and Paracrine Secretion of Vascular Endothelial Growth Factor in the Pre-Hypoxic Diabetic Retina

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Abstract: Vascular endothelial growth factor (VEGF) is well established as the main agent responsible for vascular leakage and angiogenesis in the diabetic retina. While VEGF can have positive effects on hyperglycemia stressed retinal tissues, it also plays a role in events progressing to the oxygen-stressed, *i.e.* hypoxic, diabetic retina. Some VEGF makes its way to the retina from systemic sources and some is produced locally within the eye. Hyperglycemia, oxidants, inflammation, and advanced glycation end-products are all stimulants to VEGF production, both in the hypoxic and the pre-hypoxic retina. Endothelial cells, pericytes, Müller cells, microglia, astrocytes, retinal pigment epithelium and neurons have all been known to produce VEGF at some point in retinal development or in disease. Excessive VEGF production in the early diabetic retina can lead to retinal exposure or mechanisms which exacerbate further damage. While Müller cells are likely the most significant producer of VEGF in the pre-hypoxic retina, other VEGF producing cells may also play a role due to their proximity to vessels or neurons. Study of the release of VEGF by retinal cells in hyperglycemia conditions, may help identify targets for early treatment and prevent the serious consequences of diabetic retinopathy.

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1. INTRODUCTION

Vascular endothelial growth factor A (VEGF) is a potent cytokine associated with cell-survival, mitogenesis of endothelial cells and vascular permeability [1-4]. It is found in many different tissues of the body where, depending on its concentration and balance with other cytokines, it can exert either positive or deleterious effects [2]. In the retina, suppression of VEGF has been associated with apoptosis of Müller cells (MC) and retinal neurons, including photoreceptors [5]. Recent evidence suggest anti-VEGF treatments may result in reductions in super-oxide dismutase (SOD2) in Muller cells, exposing retina to higher concentration of reactive oxygen species (ROS) [6, 7]. In humans, increased levels of hematologic VEGF have been linked to cancer, atherosclerosis, male smoking, leukocyte count, and decreased HDL-concentration [8-10]. Elevated VEGF levels have been linked to neovascular eye diseases such as diabetic retinopathy (DR) both in the early and late stages [11-13]. VEGF-B, a member of the VEGF family whose gene is located on chromosome 11, has been found to function in the maintenance of newly formed vessels during pathologies, neurons and control uptake of fatty acids into endothelial cells of the heart and skeletal system [14].

The human VEGF (VEGF-A) gene is comprised of eight exons in chromosome 6. Alternative splicing of this gene results in VEGF molecules with different peptide lengths including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$ [15].

All VEGF isoforms contain peptides coded from exons 1-5 and 8 [15]. $VEGF_{121}$ does not contain amino acids encoded by exons 6 and 7. Likewise, exon 6 does not code any amino acids in $VEGF_{165}$. While $VEGF_{189}$ contains amino acids coded by exons 1-8, $VEGF_{206}$ has an extra 17 amino acid residues encoded by exon 6 [15-17]. Of the 24 amino acids common to $VEGF_{189}$ and $VEGF_{206}$ encoded by exon 6, 12 of these are basic amino acids. Exon 7 encodes 44 amino acids in $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, 9 of these are basic. The shorter forms of VEGF are more acidic. $VEGF_{165}$ and $VEGF_{189}$ have 16 cysteines, whereas $VEGF_{206}$ has 18,

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and $VEGF_{121}$ only has 9 [16, 17]. While $VEGF_{121}$ is soluble, practically all $VEGF_{189}$ and $VEGF_{206}$ are bound to cell surfaces or the extracellular matrix (ECM). $VEGF_{165}$ has solubility properties between $VEGF_{121}$ and $VEGF_{189}$ with 50-70% of it bound to cell surfaces or the extracellular matrix [4, 18]. Splice variants $VEGF_{121,}$ $VEGF_{165}$, and $VEGF_{189}$ are the most abundant in humans [15, 16, 19]. Receptors for VEGF are found mainly on endothelial, pericyte, and bone marrow-derived cells [4, 20, 21]. VEGF receptor 1 (VEGFR-1, Flt-1) binds VEGF-A, placental growth factor (PLGF), and VEGF-B and has generally been considered to act as a decoy receptor suppressing VEGF activity, but in some cases it has been found to promote mitogenesis and to release some tissue-specific growth factors [4, 22]. VEGFR-1 is found in higher concentrations than VEGF receptor 2 (VEGFR-2, Flk-1) in retinal pericytes [23-25]. Due to its primary role in mediating VEGF responses such as angiogenesis, permeability, and mitogenesis, VEGFR-2 is considered the functional VEGF receptor. Up-regulation of both VEGF and VEGFR-2 mRNA and protein have been noted in the inner retina of streptozotocin (STZ) induced diabetic rats [26, 27]. VEGFR-3 plays a role in the development of vasculature, but in the adult it is found primarily in the lymphatic endothelium [28]. Neurophilin 1 (NP1) and 2 (NP2) also bind VEGF and appear to enhance the effects of VEGF binding to VEGFR-2. VEGF binding to NP-1 and 2 requires part of exon 7, which is not present in $VEGF_{121,}$ thus explaining the increased potency of $VEGF_{165}$ compared to $VEGF_{121}$ [4].

VEGF has different actions on retinal tissues depending on the stage of DR [29]. Many different stimuli cause increased production of VEGF in the diabetic retina. It is the goal of this review to discuss those, which occur in early diabetes, *i.e.* the pre-hypoxic retina, before VEGF production mechanisms linked to hypoxia such as Hypoxia Inducible Factor (HIF-1) play a role. Many of these actions may be harmful, but some can be protective such as VEGF protection of retinal neurons in early disease [30]. Hyperglycemia, oxidative stress, and inflammation can induce production of VEGF either from systemic sources or locally within the eye [31]. It has been proposed that advanced glycation end-product formation (AGE), activation of protein kinase C (PKC) and oxidant formation can all be initiated by superoxide production from hyperglycemia through the mitochondrial election-transport chain [31, 32]. The early stages of experimental DR require the manifestation of intercellular adhesion molecule 1 (ICAM-1) along with VEGF for vascular effects to occur, thus implicating systemic sources of VEGF in the initiation of DR [31]. In early and late diabetic retinal disease, VEGF alters tight junctions between endothelial cells, resulting in increased vascular permeability exposing the retina to systemic sources of VEGF and increasing the production of VEGF from retinal sources. As vessel damage continues in the progression of DR, hypoxia can also occur, contributing to ocular VEGF production. VEGF's mitogenic effects can result in neovascularization of the retina, *i.e.* proliferative diabetic retinopathy (PDR). VEGF is a major, but not the only, factor in diabetic macular edema (DME), which can occur at either the early or late stage of DR [33].

VEGF is produced from many ocular and non-ocular sources. Since long-term hyperglycemia enhances the release of VEGF in extra-ocular tissues, VEGF could arrive to the eye *via* vascular perfusion. Oxidized low-density lipoproteins (oxLDL) and glycated albumins, both associated with hyperglycemia, have been found to raise VEGF expression from macrophages and renal glomeruli [34-37]. Increased plasma levels of VEGF have also been correlated with S-cone dysfunctions in a population of children and adults with type 1 diabetes in which no visible signs of retinopathy were yet present [38].

There is significant confusion as to the correlation of plasma, serum and intraocular VEGF concentration. Wang *et al*. report plasma levels of individuals with proliferative diabetic retinopathy to be 3.6x higher than controls and vitreous levels to be 4.7x higher [39]. Burgos *et al*. found no differences in correlation between serum and vitreous VEGF concentrations in DR [40]. Debate has ensued over whether soluble VEGF travels in blood plasma, or whether it is stored in platelets [41]. Shlingemann *et al.* reported that platelets in type 1 diabetes patients showed no difference in their state of activation compared to normal controls. When platelet activation was inhibited during blood collection procedures, free VEGF plasma levels were low in both patients with (6.1 ± 9.5 pg/mL) and without diabetes (6.1 ± 9.4 pg/mL) and did not correlate with any diabetes markers. Using more traditional methods of blood collection, *i.e.* citrate, the plasma contained significantly higher levels of VEGF in diabetic patients (16.5 ± 12 pg/mL) than in normal controls (10.4 ± 4.9 pg/mL) and correlated with glycosylated hemoglobin levels (A1c) [36]. This suggests that patients with diabetes either carry a higher concentration of VEGF in their platelets or that diabetic conditions cause the platelets to release more VEGF. How this platelet stored VEGF is released to interact with ocular tissues has not been well clarified.

While there can be debate as to how much VEGF arrives in the retina from systemic sources much of the VEGF present in the eye during diabetes and the development of DR comes from local VEGF production [11, 12, 42, 43]. Endothelial cells, pericytes, Müller cells, microglia, retinal pigment epithelium, and neurons have all been known to produce VEGF at some point in development of disease, although there is no evidence that neurons are a significant producer of VEGF during diabetes (Fig. **1**) [44]. It is interesting to note that while most studies of VEGF concentration in the eye rely on vitreous and aqueous sampling, Velez-Montoya *et al.* found in a study of retinopathy of prematurity that sub-retinal concentrations of VEGF were 22x higher than aqueous levels, 13.8x higher than vitreous levels, and 23.5x higher than plasma levels [45]. What is less clear is where ocular VEGF production occurs in DR and actually impacts retinal changes. Murata *et al*. report VEGF mRNA markers were expressed in ganglion cells, glial cells such as astrocytes and Muller cells, smooth muscle cells and pericytes in the vessel walls, and retinal pigment epithelium. VEGF was found to be distributed along all layers of the retina. However, VEGF was most prominently noted in the nerve fiber layer [29]. Relevant factors influencing damage in diabetes are the level of VEGF release and its proximity to vessels, especially endothelial cells and pericytes.

In this review article, we summarize, for the first time, major retinal sources of VEGF in the pre-hypoxic diabetic

retina (Tables **1-4**). This information is key to the understanding of early events in the etiology of DR caused by excessive VEGF. Detailed understanding of the origins and action of VEGF at this stage will provide opportunities for clinical interventions.

2. ENDOTHELIAL CELLS AND PERICYTES

Although VEGF has been found to target other cells including pericytes and some non-vascular cells, the primary target of VEGF is the vascular endothelial cell [1, 5, 23]. Interacting together, pericytes and endothelial cells assemble the vascular basement membrane [46-48]. Endothelial cells surround the vascular lumen and are enclosed by pericytes typically being only about 20 nm apart [47]. While endothelial cells are exposed to the vascular lumen, pericytes are only associated with endothelial cells and the retinal capillary basement membrane [49]. Pericytes and endothelial cells have several connections with each other including pericyte cytoplasmic fingers inserted into endothelial cell invaginations (peg-socket type), areas where the two cells membranes come very close, and other areas where cytoplasmic filaments near the plasma membrane appear to be associated with extracellular fibronectin plaques between the two cells [47, 48]. These types of connections may vary in different tissues, but in some cases may include as many as 1,000 pericyte contacts to a single endothelial cell [48]. A single pericyte can partially cover several endothelial cells [46]. The ratio of pericytes to endothelial cells in the inner retinal capillaries is approximately 1:1; in the rat it is about 1:3 which is similar to rat cerebral capillaries, but significantly higher than other tissues such as skeletal muscle [48-50]. Pericytes cover about 40% of the circumference of normal rat retinal capillaries [50].

Pericytes are more resistant to aging and ischemia than endothelial cells. However, Kuwabara and Cogan note that diabetes is the one disease where pericytes in the retina are preferentially lost before endothelial cells [49]. In the diabetic retina a reduction of the pericyte-endothelial ratio has been linked to increased endothelial cell proliferation, a hallmark of PDR [49, 51]. Also, it has been proposed that pericytes regulate capillary proliferation [47, 49, 52].

Paracrine and juxtacrine signaling between pericytes and endothelial cells is required for vascular development and maintenance and has been extensively explored [49]. Platelet-derived growth factor (PDGF-B) is released from endothelial cells and has been found to bind to pericyte surface receptor platelet-derived growth factor receptor β (PDGFRβ). Knockouts of PDGF or its receptor result in pericyte deficiency. Collaboration between pericytes and endothelial cells is required for vascular development and maintenance [53]. Angiopoietin-1 (Ang-1) expressed by pericytes and its endothelial cell receptor Tie-2 have also been implicated in the development and maintenance of vasculature. Other cytokines such as heparin-binding epidermal growth factor (HB-EGF), stromal-derived factor 1-α (SDF-1α), chemokine C-X-C receptor 4 (CXCR4), sonic hedgehog (Shh), neurogenic locus notch homolog protein 3 (NOTCH3), jagged-1 (JAG-1), ephrin and ephrin receptor have also been found to play a role in endothelia-pericyte crosstalk and vascular stability [48]. The juxtapositional properties of pericytes and endothelial cells are illustrated by Erber *et al.* in which solid rat tumors implanted in athymic mice experienced loss of mature and immature vessels when VEGFR-2 and PDGFR-β were blocked resulting in loss of physical association between pericytes and their adjacent vascular endothelial cells. When VEGFR-2 was blocked, immature vessels quickly regressed, but in mature vessels pericytes stabilized the vessels. Pericytes were recruited to remaining immature vessels and increased expression of other survival factors such as Ang-1. It was only when the pericyte receptor PDGFR- β was also blocked that endothelial cells on mature vessels underwent apoptosis [54].

In normal physiological environments, endothelial cells cultured alone have been found to secrete very little VEGF [55]. No direct comparisons of VEGF production from different retinal cells in pre-hypoxic environments have been carried out. In a hypoxic environment, Watkins *et al*. compared the response of Muller cells, retinal pigment epithelium (RPE), astrocytes and retinal microvascular endothelial cells (EC) and found that endothelial cells contributed the least VEGF into the cellular media. Hypoxic Muller cells produced 140x more, RPE 10x more, and astrocytes produced 27x more secreted VEGF than EC [15]. When endothelial cultures were combined with pericyte precursors in a co-culture system, pericytes were found to increase their production of cell-associated VEGF and VEGF in the conditioned media [55]. Pericyte production of VEGF has also been associated with endothelial cell stability during and after vessel development [51, 55]. Endothelial cell proliferation is suppressed in cells grown in contact with pericytes, but is not inhibited when pericytes are not in contact or close proximity with the endothelial cells. Furthermore, inhibition of proliferation decreases as the ratio of endothelial cells to pericytes increases. No inhibition of growth was noted with RPE, fibroblast, or kidney epithelial cells exposed to pericytes [51].

In early diabetes, there is not yet enough retinal vessel damage to result in hypoxia of the retina. At this stage, destructive elements to the retina include hyperglycemia, advanced glycation end-products (AGE), oxidants and protein kinase C. Pericytes are lost early in diabetic retinopathy, but at an early stage they can still be producers of VEGF [56-58]. Vidro *et al.* demonstrated that high glucose treatment of human retinal pericytes in cell culture resulted in a reduction of viable cell number and an increased production of VEGF per cell from remaining viable cells. Further, these cells produced significantly more VEGF than TGFβ2 with increasing glucose concentration. These effects were lost with the addition of exogenous TGFβ2 [58].

Both intra and extracellular VEGF protein and mRNA were found to be increased with exposure to hyperglycemic media in a rhesus monkey and a rat retinal endothelial cell line and in primary bovine retinal endothelial cells [59-61]. Secreted VEGF protein was increased by 50% in response to 25nM glucose in the rat retinal endothelial cell line [59]. However, using human umbilical vein endothelial cells, Yang *et al.* demonstrated a decrease in VEGF mRNA and protein secretion with hyperglycemic media. They found that adding exogenous VEGF to the culture media was protective against apoptosis [62].

Increased VEGF production with hyperglycemia appears to be at least partially induced through transcription factor specificity protein (Sp1) [59, 63]. Increased insulin exposure has been found to increase VEGF production. Wu *et al.* found in bovine retina endothelial cells that increasing glucose levels attenuated elevated VEGF production that could be attributed to insulin exposure. In contrast, cells previously exposed to high glucose insulin exposure decreased VEGF production [60].

High glucose also acts *in vivo* through other mechanisms to increase VEGF production. One of these is the formation of advanced glycation end-products (AGE) from glucose attachment to extracellular and intracellular proteins (in cells without GLUT-4 transport) such as collagen, crystallins, and enzymes [64]. They develop both in the serum and in the retina [13]. From the serum, AGE proteins may be transcytosed into endothelial cells, bypassing the blood-retinal barrier by attachment to the receptor for advanced glycation end-products (RAGE) and deposited in the vessel wall containing the endothelial cells and pericytes. AGE proteins have been found to increase the production of VEGF mRNA and protein in bovine pericytes *in vitro* and lead to their apoptosis [56, 57, 65]. Therefore, the increased production of VEGF from pericytes in diabetes may be significant for early diabetes effects, but may be short-lived due to the imminent demise of pericytes. Increased VEGF mRNA and protein production obtained from lysed endothelial cells has also been linked to increasing AGE exposure [13, 57, 66]. The sulfonylurea and anti-oxidant gliclazide was found to decrease both VEGF mRNA and protein in these AGE-exposed endothelial cells. Other anti-oxidants vitamin E and N-acetylcysteine (NAC) were also found to decrease VEGF mRNA. Another sulfonylurea glyberide demonstrated no effect on VEGF production in AGE-exposed endothelial cells [13]. Gliclazide, vitamin E and NAC inhibited protein kinase C translocation from the cytosol to membrane fractions in these AGE-exposed cells and gliclazide reduced nuclear factor kappa B (NF-κB) binding to the VEGF pro-

Table 1. Endothelial cell VEGF production Pre-hypoxia.

Stimulus	Condition	Major Findings	References
Co-culture	*In vitro*	Co-Culture of endothelial cells and pericyte precursors; very little VEGF production compared to pericytes	Darland *et al.*, 2003
Hyperglycemia	*In vitro*	Protein lysates, secreted, and mRNA of VEGF were increased using hyperglycemic media in rhesus monkey, rat cell lines, and bovine primary endothelial cells. In HUVEC cells, VEGF seemed to protect against apoptosis, but VEGF mRNA and secreted protein were reduced with higher concentration.	Wu *et al*., 2010; Li *et al*., 2012; Donovan *et al*., 2014; Betts-Obregon *et al.,* 2016 Yang *et al*., 2008
AGE	*In vitro*	AGE exposure increased VEGF mRNA and protein levels in endothelial cell lysates.	Mamputu *et al*., 2002; Mamputu, *et al*., 2004; Shimizu *et al*., 2011; Shimizu *et al.*, 2013
ROS	*In vitro*	Anti-oxidants inhibit AGE induced VEGF mRNA increases in bovine retinal endothelial cells	Mamputu *et al*., 2002
Estrogen	*In vitro*	Estrogen induced a decrease in VEGF and PEDF protein secretion from rhesus monkey retinal endothelial cells in a dose dependent manner.	Grigsby *et al*., 2011

Table 2. Pericyte VEGF production Pre-hypoxia.

Stimulus	Condition	Major Findings	References
Co-culture	*In vitro*	Co-culture of endothelial cells (EC) and pericyte precursors resulted in a time dependent increase in VEGF secretion. Co-Culture VEGF production is mediated by TGF β. Inhibition of VEGF increased EC apoptosis. These findings suggest that pericyte-derived VEGF is required for EC survival and stability.	Darland *et al.*, 2003
Hyperglycemia	*In vitro*	High glucose decreased viable human pericyte cell number, but increased VEGF secretion into cell media (and per cell) in a concentration dependent manner.	Vidro *et al*., 2008
AGE	*In vitro*	Bovine retinal pericytes treated with different AGE products showed increased VEGF mRNA expression in a time dependent manner.	Yamagishi *et al*., 2002
		Human peripheral nerve pericytes treated with different concentrations of AGEs showed increased VEGF expression.	Shimizu *et al*., 2011
		Human brain pericytes exposed to AGEs showed no concentration dependent change in VEGF protein level.	Shimizu *et al*., 2013

moter. Thus, AGE stimulated expression of VEGF appears to proceed through an oxidative stress induced protein kinase C dependent NF-κB binding mechanism [13].

3. MÜLLER CELLS

Müller cells extend from vitreous to the outer nuclear layer, enveloping neurons and blood vessels with their cellular processes serving as an anatomical and functional link between retinal neurons and other compartments [67, 68]. Müller cells maintain the homeostasis of the extracellular space composition, aid in recycling of neurotransmitters (namely glutamate and GABA), release gliotransmitters (principally glutamate, ATP, and adenosine), provide trophic factors to neurons, maintain the inner blood-retinal barrier, regulate the retinal blood flow, provide anti-oxidative support to neurons, act as optical fibers, regenerate 11-cis retinol during bleaching of cone photoreceptors, buffer potassium, form neural stem cells in response to injury, produce pro-inflammatory cytokines in response to infection and phagocytize fragments of retinal cells and foreign particles [67, 69-72].

Müller cells are modulators of angiogenesis in proliferative diabetic retinopathy. They secrete PEDF, a potent inhibitor of angiogenesis, as well as VEGF, a potent angiogenesis inducer [73, 74]. Through a balance of VEGF and PEDF, Müller cells are potentially key regulators of neovascularization of the retina [75].

The importance of Müller cell VEGF production was confirmed in conditional Muller cell VEGF KO mice generated by mating transgenic Cre recombinase mice with floxed VEGF mice [76]. This results in a partial (40-60%) reduction in VEGF secretion by Müller cells. In VEGF-KO mice the development of the choroid or retina was unaffected. However, when challenged with oxygen-induced retinopathy, VEGF-KO mice showed a 43.3% reduction in ischemia-induced retinal neovascularization (NV), a 29.6% reduction of vaso-obliteration, a 33.7% reduction in NV and 35% more of the tight-junction protein occludin in comparison to WT controls [77]. In a study by Wang *et al.* both WT mice and VEGF-KO mice were made diabetic by streptozotocin (STZ) injection. Controls were untreated WT or VEGF-KO [44]. Even though VEGF expression by Müller cells was only partially ablated, immunoblotting of extracts from dissected retina and vitreous demonstrated a significant reduction (47.4%) of retinal VEGF levels in diabetic KO mice relative to WT mice. WT diabetic mice lost tight junction proteins while VEGF-KO mice retained normal levels. The VEGF-KO mice also showed reduced evidence of acellular capillaries, leukostasis, inflammatory biomarkers (ICAM-1, TNF-α and phosphorylated NF-κB p65 subunit) and vascular leakage [44]. Thus, Müller cell VEGF plays an essential part in retinal inflammation during the course of diabetic retinopathy. Four VEGF variants are known to occur in Müller cells [78]. It was concluded that the predominant type of VEGF produced by mice Müller cells is $VEGF_{164}$ [10, 69, 70].

Immuno-histologic analysis of human and rat retinas show that Müller cells are rich in VEGF receptors. Furthermore, RT-PCR analysis of purified human Müller cells as well as primary rat Müller cells has revealed mRNA for VEGFR-1 and VEGFR-2 [79, 80]. Human Müller cells also express mRNA for the isoform-specific co-receptor for VEGF-A neuropilin-1 (NP1) [80]. Müller cells that express VEGFR-1 or 2 respond to VEGF via a MAPK pathway. An antagonist to VEGFR-1 and 2, SU5416, inhibited retinal neovascularization [81]. However, adenoviral expression of sFlt1 (VEGFR-1) neutralized VEGF initiated apoptosis of cells in the INL and ONL and reduced a- and b-wave amplitudes of the ERG [5]. To assess autocrine signaling through VEGFR-2, MIO-M1 Müller cells were used. MIO-M1 is a spontaneously immortalized human Muller cell line which retains the normal Müller cell phenotype [82]. When siRNA was used to suppress VEGF by 94% in these cells, there was a doubling of Müller cell apoptosis compared to untreated controls. PCR showed that inhibition of VEGF expression up-regulated expression of the pro-apoptotic gene Bax but the expression of anti-apoptotic genes Bcl-2 remained unchanged. Thus the Bax/Bcl-2 ratio was increased. These results confirm constitutive expression of VEGF is needed not only for survival of photoreceptor and retinal ganglion cells, but also for survival of Müller cells themselves [5]. In a recent study of streptotocin-treated conditional VEGFR-2 KO mice, loss of the VEGFR-2 led to a decreased census of Müller cells, reductions in ERG amplitude and loss of rod and cone photoreceptors [30].

Most studies have found that hyperglycemia increases secretion of VEGF in Muller cell lines [83]. Using hyperglycemia with MIO-M1 cells Aldarwesh *et al.* found a statistically significant increase in VEGF mRNA at 24 hours compared to normal glucose. Secreted VEGF into the conditioned media measured by ELISA increased at 24 and 48 hours, but did not achieve statistical significance until 72 hours [83]. Vellanki *et al.* found an increase in cell number, but an insignificant difference in per cell VEGF secretion at 24 hours with both MIO-M1 and rMC-1 cells comparing physiological (5.5 nM) to high (30 mM) glucose [84]. Müller cell VEGF expression is increased early in humans during non-proliferative DR [85]. Aquaporin 4 (AQP4), a Müller cell and astrocyte channel protein and the ratio of AQP4 to aquaporin 1 (AQPA1), another glial cell channel protein, is significantly increased in the aqueous humor of humans with pre-clinical DR, indicating early Müller cell activation [86]. The mechanisms by which hyperglycemia causes gliosis and up-regulates VEGF are currently under investigation in Muller cells. A study by Schrufer *et al.* using rat and human Müller cell lines (TR-MUL and MIO-M1) showed that an increase in VEGF is due to a cap-independent mRNA translation mediated by increased expression of and decreased phosphorylation of eukaryotic initiation factor 4E (4E-BP1). This is achieved by an increased sequestration of eIF4 by 4E-BP1. Ye *et al.* reported that after Müller cells are stimulated with high glucose, phosphorylation of ERK1/2 occurs. Thus, the ERK1/2 pathway might also be involved in the increase of VEGF secretion observed in hyperglycemia. Sun *et al.* implicated X-linked inhibitor of apoptosis protein (XIAP) as a factor in increased expression of Muller cell VEGF in hyperglycemia by proving that embelin, a XIAP inhibitor, decreased VEGF expression in cultured rat Müller cells under hyperglycemic conditions. The authors suggested that XIAP may regulate VEGF through a regulatory network involving NFκB p65, p38, TNF-α, uPA, CREB, IL-1β, ERα, Stat3, and HCAM [87]. A study by Devi *et al.* suggested that

Müller cells exposed to hyperglycemia increase their VEGF secretion through a signaling pathway involving autophagy. The authors proved that rMC-1 cells exposed chronically to high glucose conditions increase their expression of TXNIP, a pro-oxidative stress protein. Sustained high glucose conditions lead to an increase in ROS, leading to ER stress as well as an increase in HIF-1α levels accompanied by a decrease of ATP. However, cell viability did not increase with high glucose conditions. Therefore, autophagy was activated as a cell survival mechanism. Excessive and continuous autophagy induces cell apoptosis, which induces Müller cells to express pro-inflammatory genes, including VEGF [88]. Zhong, Y., *et al*. found that ER stress induced by hyperglycemia also leads to the activation of activating transcription factor 4 (ATF4) that results in elevated ICAM-1 and VEGF proteins in Müller cells through a pathway involving c-Jun N-terminal Kinases (JNK) [89].

Dennis *et al.* found that hyperglycemia increased both VEGF and a ubiquitous protein regulated in DNA damage and development (REDD1) in retinal homogenates from STZ-treated rats with the effects cancelled by normalizing serum glucose with the anti-oxidant and glucose receptor inhibitor, phlorizin. In hyperglycemic Müller cells (human MIO-M1 and rat TR-MUL), REDD1 plays a permissive role in VEGF translation by repressing akt/mTORC1 phosphorylation of 4E-BP1, allowing it to dissociate from the cap-binding protein elF4E and slow cap-dependent translation. Cap-independent translation then ensues to augment VEGF production. After 4 weeks of STZ-treatment wild type mice showed increased expression of VEGF and the pro-inflammatory TNF-α. These increases did not occur in REDD1-KO mice. Thus REDD1 is necessary for hyperglycemic increases in Müller cell VEGF [90].

In MCT cells, a mouse renal proximal tubule cell line, it has been shown that angiotensin II promotes 4E-BP1 phosphorylation, thus up-regulating VEGF translation. Furthermore, hyperglycemic conditions increase synthesis of angiotensinogen, a precursor of angiotensin. Thus, in MCT cells, hyperglycemia might lead to an increase in angiotension II causing more 4E-BP1 phosphorylation and thus more VEGF expression [91, 92]. Müller cells have angiotensin receptors and both angiotensin converting enzyme (ACE) and angiotensin II are elevated in vitreous samples of patients with diabetic retinopathy. Moreover, cultured human Müller cells contain mRNA for renin, angiotensinogen, ACE and angiotensin receptor type 1 [93]. Both valsartan, an angiotensin I receptor antagonist, and PD123319, an angiotensin II receptor antagonist, decreased VEGF expression [94].

Advanced Glycation end-products (AGE) result from hyperglycemia and are associated with diabetic retinopathy in human patients and animal models of experimental diabetes. AGEs worsen oxidative stress and inflammation by interacting with RAGE receptors on susceptible cells, including Müller cells [95-97]. Müller cell VEGF expression and secretion follows RAGE activation. Early reports showed a relationship between AGEs and Müller cell VEGF. PCR and western blots from cultured Müller cells showed that VEGF mRNA and protein were increased by AGEs. Vitreous sampled from PDR patients showed significantly higher AGE and VEGF than vitreous from controls without diabetes [95]. Pathogenic effects of diabetes in STZ-treated rats were blocked by pyridoxamine, an inhibitor of AGEs and advanced lipoxidation end products (ALEs) [98].

In a study of hyperglycemic and hyperlipidemic mice, ERG, immunohistochemistry, RT-PCR, autoFL and ELISA were employed to study AGE and RAGE. AGEs were associated with the footplates of RAGE-positive Müller cells and were increased in the extracellular matrix in hyperglycemic mice. RAGE mRNA transcripts were highest in High-glucose High Lipidemic mice compared to normal mice. RAGE-induced formation of acellular capillaries and pericyte ghosts was reduced by application of soluble RAGE (sRAGE) that competitively inhibits RAGE function [99]. AGE, ALE, giant fibrillary acidic protein (GFAP) and heme-oxidase were studied in normal control rats, diabetic rats (via STZ) and diabetic rats w/pyridoxamine. Pyridoxamine treatment achieved significant reduction in AGEs, ALEs, GFAP and heme-oxidase in ganglion cells and the inner plexiform layer (IPL); moreover, abnormal positioning of the K^+ channel protein (Kir4.1) and aquaporins were also reduced by pyridoxamine [96].

Zong *et al.* (2010) studied RAGE, the RAGE ligand S100B and GFAP in retinal cryosections of STZ and control rats. RAGE increased in the experimental diabetes. They also exposed M10-M1 Muller cells to normal and 5x glucose media, with the latter causing increased GFAP, RAGE and S100B. Use of sRAGE or a si-RAGE protocol blocked VEGF increases observed in high glucose. It was also suggested that RAGE activates a MAPK pathway [97]. In a related study, STZ-treated and normal rats fed a normal diet were compared to the same groups fed a high fat diet. The number of pericytes declined in both diabetic groups, but in the diabetic rats fed a high fat diet, markers for diabetic complication (RAGE, GFAP, VEGF and TNF-α) increased significantly [100].

Inevitably, a $RAGE^{-/-}$ mouse was developed for comparison to wild-type (WT) mice. Both were given either sham saline injections or injections of STZ. The WT mice developed DR and increased concentrations of RAGE at 12 weeks but not at 24 weeks. GFAP was increased in WT, but importantly, RAGE -/- mice were protected from this effect [101]. Clearly, RAGE binding by AGE ligands in experimental diabetes activates Muller cells as evidenced by increased secretion of GFAP and VEGF. Potential treatments can now include inhibition of the AGE/RAGE signaling pathway.

4. MICROGLIA AND ASTROCYTES

Microglia are the resident macrophage of the retina. Their interaction with other retinal cells has been postulated to play a key role in DR [102]. In early diabetes circulating VEGF production and local production of ICAM-1 and VCAM can increase the exposure of microglia to inflammatory cytokines transitioning microglia into an activated, *i.e.* amoeboid, state [102-104]. High glucose, AGE, or ROS can perpetuate this state.

De-Oliveria-Simoes-Pereria *et al.* found that when rat retinal cells were cultured in high glucose, purinergic P2 receptors in microglia were up-regulated, causing a calcium influx and release of microglial associated pro-inflammatory

Table 3. Müller cell VEGF production Pre-hypoxia.

Stimulus	Condition	Major Findings	References
Hyperglycemia	*In vitro*	Hyperglycemic treatment for 24 hours resulted in a 4-fold increase in VEGF mRNA and a 2.6 fold increase in secreted protein production at 72 hours using MIO-M1 (human Müller cell line) and retinal explants.	Alderwash *et al*., 2016
	In vitro	Hyperglycemia increased MIO-M1cell number, but no significant change in per cell VEGF secretion at 24 hours with MIO-M1 or rat (rMC-1) cells.	Vellanki *et al*., 2016
	In vitro	In cultured primary rat Müller cells, hyperglycemia elevated VEGF secretion. This expression was reduced by an inhibitor-U0126. U0126 stopped phosphorylation of ERK ½.	Ye *et al*., 2012
	In vitro	Embelin an inhibitor of XIAP (blocks cells from entering apoptosis), normalized the increased VEGF protein production in rat Müller cells attributed to hyperglycemia.	Sun *et al.*, 2013
	In vivo and *in vitro*	VEGF mRNA levels and HIF-1α activity were increased in a rat Müller cell line due to hyperglycemia, this change was linked to thioredoxin interacting protein (TXNIP)	Devi *et al.*, 2012
	In vivo and *in vitro*	Regulated in development and DNA damage 1 (REDD1) was necessary for hyperglycemia induced elevated VEGF protein in STZ rats and mRNA in rat TR-MUL cells.	Dennis *et al*., 2014
	In vivo and *in vitro*	Inhibition of activating transcription factor 4 (ATF4) in rat Müller cell line (rMC-1) and C57BL/6 J mice decreased VEGF production.	Zhong *et al*., 2011
	In vivo	Frozen sections of eyes with diabetes, but without PDR showed VEGF expression in Müller cell distribution.	Amin *et al*., 1997
	In vivo	Early activation of Müller cells is shown in pre-clinical DR.	Vujosevic *et al*., 2015
	In vivo and *in vitro*	Müller cell-VEGF mRNA expression was increased early (3 weeks) in STZ rats through cap-independent translation. Similar results were observed in cultured Müller cells.	Schrufer *et al.*, 2010
Angiotensin/ Hyperglycemia	*In vivo* and *in vitro*	Hyperglycemia upregulates angiotensinogen. Angiotensin II promotes 4E-BP1 phosphorylation which upregulates VEGF. VEGF expression was decreased with angiotensin I and II receptor antagonist in rat retina.	Kida *et al.*, 2003; Zhang *et al.*, 2004; Schrufer *et al.*, 2010; Ye *et al.*, 2012
AGE	*In vivo* and *in vitro*	AGE increased VEGF mRNA and protein expression in cultured Müller cells. Pyridoxamine (AGE inhibitor) blocked development of retinopathy in diabetic rats Elevated VEGF levels in hyperglycemia were inhibited by blocking/absence of RAGE.	Hirata *et al*., 1997; Stitt *et al.*, 2002; Zong *et al*., 2010; McVicar *et al.*, 2015;

mediators seen in DR [105]. Molecules released by activated retinal microglia include glutamate, proteases, leukotrienes, IL-1β, IL-3, IL-6, TNF-α, VEGF, lymphotoxin, macrophage inflammatory protein 1 (MIP-1), matrix metalloproteinases (MMPs), caspase 3, and ROS [103, 106]. TNF-α leads to the production of ICAM-1 and VCAM thus further contributing to retinal vascular leakage and creating a circle of inflammation and VEGF production [107].

Astrocytes (AC) migrate from the optic nerve head during development. Throughout life, they are found in the retinal nerve fiber and ganglion cell layers and the intraocular portion of the optic nerve [86, 108-111]. During development, astrocytes produce VEGF and may play some role in inner retinal vasculature formation [112-115]. Adult mammal AC are densely packed near blood vessels. They serve several functions such as regulation of blood flow, conversion of glucose to lactate, and removal of toxins from retinal neurons [116, 117]. Their density is dependent on the thickness of the nerve fiber layer, being higher in the superior and inferior arcades and less at the macula and the ora [110]. It is believed that astrocytes play a role in stabilizing retinal vasculature, but in response to hypoxia HIF-1 stimulate VEGF production in the adult, creating pathological neovascularization such as in PDR [112, 113, 115]. It is well established that astrocytes produce VEGF during diabetic retinopathy [29, 118]. What is less clear is that stimuli other than hypoxia may also activate astrocyte dysfunction and VEGF production. Using hyperglycemia as a stimulus, Shin *et al.* found increases in inflammatory cytokines and activation of pathways linked to VEGF production, but they did not specifically test for VEGF mRNA or protein production [119-121].

5. RETINAL PIGMENT EPITHELIAL CELLS

The retinal pigment epithelium (RPE) is a monolayer of cells that constitute the outer blood-retinal barrier (BRB). The main functions of RPE cells are the following: supply nutrients to photoreceptors and maintain ion homeostasis in the sub-retinal space, absorb scattered light in order to protect against photooxidation, re-isomerize all-trans-retinol into 11-cis-retinal as part of the visual cycle, phagocytize shed photoreceptor membranes, secrete a variety of signaling molecules essential for the structural integrity of the retina and maintain the inner eye as an immune privileged space [59, 122-125]. RPE cells are capable of acting as oxygen and

metabolic sensors that regulate angiogenesis in response to changing local environment [122]. It is known that the production of VEGF by RPE cells constitutes an important step for the development of DR, clarifying the stimulus for its up-regulation is extremely important in understanding DR pathogenesis.

It is known that hyperglycemia instigates a series of biochemical changes that ultimately lead to vascular dysfunction, increasing vascular permeability and capillary rarefaction (a decrease in the number of perfused capillaries in an area of tissue). In ARPE-19, a human RPE cell line, it has been demonstrated that hyperglycemic conditions affect cell growth, cell differentiation and cell functions. RPE cells cultured in 18mM glucose proliferate at a considerably faster rate than cells cultured in euglycemic conditions and assume a more elongated shape [126]. Hyperglycemia itself is considered a potent stimulus for VEGF secretion by RPE. It has been demonstrated that culturing primary human RPE or ARPE-19 cells in high glucose up-regulates VEGF mRNA and protein expression [123, 126]. Chang *et al.* suggested that high glucose conditions induce carbohydrate response element binding protein (ChREBP) to activate HIF-1, leading to the increased VEGF expression [124]. Additionally, Vogt *et al.* reported that ARPE-19 cells cultured in high glucose conditions increased bone morphogenetic protein 4 (BMP-4) secretion and that BMP-4 increased VEGF secretion through unknown transduction pathways [127]. Furthermore, Wang *et al*. demonstrated that RPE cells up-regulate integrin-linked kinase (ILK) in high glucose conditions and proposed that the ILK pathway may be involved in the response of RPE cells to hyperglycemia [125].

Even though several pathways seem to influence VEGF hyperglycemia mediated induction in RPE cells, the main pathway involved may be the O-linked b,N-acetylglucosamine (O-GlcNAc) posttranslational modification and its effect on the transcription factor Sp1. O-GlcNAc glycosylation promotes Sp1 stability, localization to the nucleus and transcriptional activity, resulting in increased VEGF expression. Because O-GlcNac is produced by the hexosamine biosynthetic pathway, O-GlcNac glycosylation and Sp1 activity are up-regulated in hyperglycemic conditions. Donovan *et al.* managed to successfully abrogate VEGF up-regulation in ARPE-19 cells by inhibiting O-GlcNAc posttranslational modification of Sp1. Thus, the proposed mechanism for VEGF-A up-regulation in hyperglycemic conditions is the increase in Sp1 activity through the pan-cellular increase of O-GlcNAc glycosylation. Significantly, RPE cells in which O-GlcNAc glycosylation or Sp1 transcription factor are inhibited are glucose insensitive but do not decrease the basal VEGF-A production, making inhibitors of the enzyme O-GlcNAc transferase an attractive potential therapy for diabetic retinopathy [59].

High glucose conditions may not induce VEGF expression directly as potently as the other biochemical changes. One such metabolic alteration is the production of AGEs. It has been demonstrated that AGEs induce RPE cells to up-regulate several cytokines and growth factors, such as VEGF, IL-8 and monocyte chemoattractant protein - 1 (MCP-1), which might be important for DR pathogenesis [128, 129]. Incubation of human RPE with AGEs alone results in a 5-fold increase of VEGF mRNA production. This increase in VEGF mRNA by AGE exposure is blocked by the antioxidants N,N9-dimethylthiourea (DMTU) and N-acetylcysteine (NAC), implicating reactive oxygen intermediates (ROI) as mediators for this response [130] Additionally, the RAGE-mediated up-regulation of VEGF secretion by ARPE-19 cells seems to be largely dependent on NF-κB [131].

Metabolic abnormalities found in chronic hyperglycemia are ROS production, increased flux through the polyol pathway, and excessive protein glycation [132]. RPE cells treated with either tert-butylhydroperoxide (tBH), buthionine sulfoxime (BSO) or oxidized low density lipoprotein (oxLDL) and thus under oxidative stress have shown an increase of VEGF secretion [133-137]. Furthermore, on ARPE-19 cells treated with 4-hydroxynonenal (HNE) or malondialdehyde (MDA), it was observed that lipid peroxidation products caused an increase of total VEGF-A protein secretion into the culture medium [138]. Li *et al.* proposed that the high VEGF secretion observed in high glucose conditions was due to the oxidative stress induced in RPE cells by hyperglycemia that activated the JAK-2/STAT3 pathway [139].

Not only does oxidative stress induce VEGF expression in RPE cells, but it also contributes to inflammation and dysregulation of the complement system. In ARPE-19 cells with increased intracellular ROS levels induced by cigarette smoke concentrate (CSC) or H_2O_2, premature senescence with increased VEGF, IL-6 and IL-8 expression and down-regulated complement factor H (CFH) were demonstrated [140]. Similarly, hydroquinone-treated human RPE cultures exhibited, apart from increased levels of VEGF, higher IL-12 and IL-10 levels and lower MCP1 and pigment epithelial derived factor (PEDF) levels [141, 142]. Another cigarette smoke component acrolein, an aldehyde, has also been found to increase VEGF production in ARPE-19 cells [143]. Thurman *et al*., propose that since oxidatively stressed ARPE-19 cells show decreased levels of complement decay-accelerating factor (DAF), CD59 and CFH, oxidative stress induction of VEGF expression might be mediated through complement activation [144]. Additionally, complement activation in RPE cells increases MMP-2 and MMP-3 that further increase VEGF and decrease PEDF [145].

Chronic inflammation observed in patients with DR is a key component of pathologic neovascularization [122]. Protein and mRNA levels of VEGF in human RPE cells incubated for 24 h with IL-1β (a pro-inflammatory molecule) were up-regulated two- to four-fold. This was significantly suppressed by adding IL-4 (an anti-inflammation factor) [123]. Likewise, the inflammatory molecules IFNγ, IL-6 and TNFα have been shown to increase ARPE-19 VEGF secretion by 3.9, 1.22, and 1.82X, respectively [146]. A study using a mix of inflammatory cytokines containing IFNγ, TNF-α and IL-1β demonstrated that inflammation not only increases VEGF-A secretion in human RPE cells by 10-fold but also increases VEGF-C expression by 9-fold [147].

In summary, hyperglycemia seems to be the main driving force for the up-regulation of VEGF secretion by RPE cells in pre-hypoxic DR by direct and indirect mechanisms.

Table 4. Retinal pigment epithelial cell VEGF production Pre-hypoxia.

Stimulus	Condition	Major Findings	References
Hyperglycemia	*In vitro*	mRNA and protein levels of VEGF were increased in hRPE cells incubated in hyperglycemic conditions. Such response was shown to be suppressed by addition of IL-4 or melanin and by knocking down ILK gene expression. Additionally, the O-GlcNAcylated transcription factor Sp1 seemed to be involved.	Klettner *et al.*, 2015; Wang *et al*., 2012; Donovan *et al.,* 2014; Grigsby *et al*., 2012; Heimsath *et al.,* 2006
AGE	*In vivo* and *in vitro*	Injection of AGEs in rat and rabbit eyes increased VEGF mRNA levels. Also, hRPE cultured with AGEs up-regulated VEGF protein expression in a time and dose dependent manner. This expression was further blocked by antioxidants DMTU and NAC.	Lu *et al.*, 1998
Oxidative Stress	*In vitro*	In hRPE cells, VEGF expression was up-regulated following exposure to TBHP, BSO, hydroquinone, high glucose conditions (resulting in ROS production), and H_2O_2 with human serum (as a source of complement proteins). ARPE-19 and porcine RPE cells exposed to tBH increased VEGF expression, possibly through p38 and ERK pathways. In APRE-19 cells, addition of oxLDL, bafilomycin A1 (to block lysosomal function), lipid peroxidation products HNE and MDA and H_2O_2 followed by CSC (as inductors of senescence) resulted in VEGF up-regulation.	Rabin *et al*., 2013; Sreekumar *et al*., 2006; Li *et al*., 2012; Cao *et al.*, 2013; Pons *et al.*, 2011; Thurman *et al.,* 2009; Kannan *et al.*, 2006; Klettner *et al*., 2009; Li *et al*., 2014; Bergmann *et al.*, 2011; Marazita *et al*., 2016
Inflammatory proteins	*In vitro*	In hRPE and ARPE-19 cells, IFNγ, IL-6, and TGFβ increased VEGF mRNA expression. The pathway involved in INFγ up-regulation of VEGF is dependent on PI3K/mTOR. In hRPE cells, the exposure to a mix of IFN-γ + TNFα + IL-1β resulted in an increase of VEGF and up-regulation of NF-κB and JAK-STAT pathways. Furthermore, addition of TGF-β1, TGF-β2, TGF-β3, IL-1ß, TNF-α and MMP-9 increased VEGF secretion. ARPE-19 cells exposed to C5a up-regulate VEGF secretion.	Liu *et al.*, 2010; Nagineni *et al.*, 2012; Cortright *et al.*, 2009; Bian *et al.*, 2007; Nagineni *et al.*, 2003; Hollborn *et al.*, 2007
Coagulation factors	*In vitro*	In hRPE cells, FXa and thrombin upregulated VEGF mRNA and protein expression.	Hollborn *et al.*, 2012; Bian *et al.*, 2007
Insulin	*In vivo* and *in vitro*	Insulin increased VEGF mRNA levels in rat RPE cell layers, and in cultured hRPE.	Lu *et al.*, 1999

6. *IN-VITRO* VEGF PRODUCTION AND SECRETION BY RETINAL CELLS

It is of particular interest to distinguish between VEGF production which includes mRNA and protein synthesis and VEGF secretion, which denotes the release of synthesized VEGF from VEGF producing cells (Fig. **1**). Although many reports focus on VEGF synthesis by retinal cells *in vivo* and in *vitro*, only a few references have provided quantitative insights on VEGF secretion. The latter is rather difficult to assess *in-vivo* as secreted VEGF is quickly diffused from source to target or removed by circulation. However, the secretion of VEGF can be readily accessed *in-vitro* by examining VEGF levels in cell media of cultured retinal cells. Table **5** summarizes data on the *in-vitro* secretion of VEGF from four types of retinal cells grown in normal and high glucose for 24 hours. Consistent with reports on retinal VEGF producing cells, Müller cell and RPE secreted higher levels of VEGF (fg VEGF per cell) than retinal endothelial cells and retinal pericytes in both normal (5.5mM) and in high (18 or 30mM) glucose conditions (Table **5**). Although high glucose induced a significant increase (from 40-185%) in VEGF secretion (pgVEGF/cell) from retinal RPE, retinal endothelial cells and pericytes, at 24 hours, it did not induce an increase in VEGF secretion per Müller cell. This is a novel observation which requires further investigation as high glucose is associated with higher levels of VEGF synthesis in Müller cells (see Table **3**).

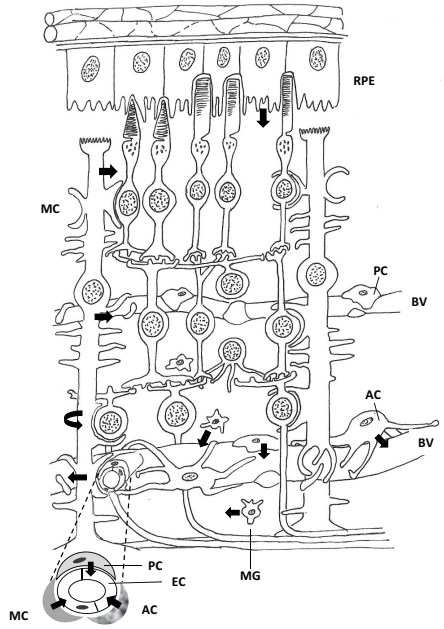

Fig. (1). VEGF-secreting cells within the retina. VEGF, indicated by ➨. *Legend:* **RPE** – Retinal Pigment Epithelium **PC** - Pericyte, **AC** – Astrocyte, **MG** – Microglia, **EC** - Endothelial Cell, **MC** – Muller Cell, **BV** – Capillary.

Table 5. ***In vitro*** **VEGF secretion (fgVEGF/cell) into the conditioned media by retinal cells in normal glucose (NG; 5.5 nM) and in high glucose (HG; 18 or 30 mM). Conditioned media were collected after incubation in NG or HG for 24 hours. VEGF was measured by ELISA.**

Retinal Cell Type	Cultured Cell Source	VEGF Secretion (fg/cell) in NG	VEGF Secretion (fg/cell) in HG	% change of VEGF per cell (NG to HG)
Human Retinal Pericytes (HRP) [148]	Primary	1	2	100
Human Retinal Pigment Epithelium (ARPE-19) [126]	Spontaneously Transformed	5	7	40
Rhesus Monkey Retinal Endothelial Cells (RhREC) [61]	Spontaneously Transformed	0.7	2	185
Human Müller Cells (MIO-M1) [84]	Spontaneously Transformed	7.3	6.1	-20`

(` not statistically significant)

CONCLUSION

The synthesis and secretion of VEGF is an integral part in the process of DR and occurs for multiple reasons. While typically associated with angiogenesis and hypoxia, VEGF is synthesized and secreted by multiple retinal cell types in both the early and late stages of DR. One must remember that VEGF secretion related to hyperglycemia, AGE and ROS still occur during the hypoxic stages of DR adding to the total VEGF load during PDR. Excellent work by Watkins *et al*. compare and quantify VEGF production in various cells during hypoxic stimuli, but no such work exists of VEGF production of retinal cells during hyperglycemia, AGE, PKC stimulation, or ROS [15]. Few doubts exist that even at these early non-hypoxic stages of DR that Muller cells are a significant source of VEGF. Proximity of cells certainly plays a role in the actions they confer. Pericytes are most closely associated with endothelial cells, but may be short lived in DR development, thus the paracrine secretions of other retinal cells are important [48, 49].

Retinal pigment epithelium is well known as a major producer of VEGF in age-related macular degeneration. It has also been found to produce VEGF in diabetes in response to hyperglycemia, AGE, ROS and hypoxia. Since most of the anoxia in DR is in the inner retina and RPE is located near choroidal circulation a question remains, does RPE significantly increase its VEGF production beyond non-hypoxic levels as hypoxia becomes a factor in the diabetic retina?

The addition of estrogen into the normoglycemic conditioned media of rhesus retinal endothelial cells decreases the ratio of PEDF/VEGF secretion into the media [149]. If similar effects can be found on estrogen effects of hyperglycemia on PEDF/VEGF secretion of retinal endothelial cells it may offer an explanation as to the worsening of DR during pregnancy. Human retina contains estrogen receptors [150]. The temporary progression of DR during pregnancy is well established; 6.3% of those with mild NPDR and 29% of those with moderate NPDR will develop PDR during pregnancy [151]. Pre-puberty seems to offer somewhat of a grace period to the development of DR and males seem to have higher incidences of advanced retinopathy [149, 152]. It appears that sex hormones play a role in DR, but how they affect VEGF production *in vivo* is not clear [149, 153].

Hyperglycemia appears to be the trigger that sets the whole process in motion and AGE, PKC phosphorylation, osmolarity changes and ROS exposure evolve from it. While the levels of VEGF produced by endothelial cells, pericytes, astrocytes, microglia, Müller cells and RPE are less in the non-hypoxic retina than in hypoxia all respond to hyperglycemia-associated events and contribute to increases in VEGF [11, 83]. VEGF secretion may be protective to some retinal cells, but it also may subsequently contribute to the cumulative damage from hyperglycemia. The increased permeability resulting from retinal VEGF in early diabetes exposes the retina to AGE, ROS and inflammatory molecules that presage more severe stages of DR [31, 102]. While much work has been done in this area, many questions persist.

Information on VEGF production in the retina may lead to new approaches on treatment and intervention of neovascular diseases in the retina including DR. For example, novel and effective pharmacological interventions may be directed toward attenuating the production of VEGF from major producers such as Muller cells and pigment epithelial cells. Detailed biochemistry on the molecular pathways of VEGF production will provide invaluable opportunities to investigate inhibition of cellular processes leading to VEGF production and release under pathological conditions.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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