Published in final edited form as:

Exp Eye Res. 2013 November ; 116: 240–246. doi:10.1016/j.exer.2013.09.014.

Hypoxia-Induced Expression of VEGF Splice Variants and Protein in Four Retinal Cell Types

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Abstract

The purpose of this study was to investigate the hypoxia-induced *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ mRNA expression profiles in rat Müller cells (MC), astrocytes, retinal pigmented epithelial cells (RPE) and retinal microvascular endothelial cells (RMEC) and correlate these findings to VEGF secreted protein. Cultured cells were exposed to normoxia or hypoxia. Total RNA was isolated from cell lysates and *Vegf* splice variant mRNA copy numbers were assayed by a validated qRT-PCR external calibration curve method. mRNA copy numbers were normalized to input total RNA. Conditioned medium was collected from cells and assayed for total VEGF protein by ELISA. Hypoxia increased total *Vegf* mRNA and secreted protein in all the retinal cell types, with the highest levels observed in MC and astrocytes ranking second. Total *Vegf* mRNA levels in hypoxic RPE and RMEC were comparable; however, the greatest hypoxic induction of each *Vegf* splice variant mRNA was observed in RMEC. RPE and RMEC ranked 3rd and 4th respectively, in terms of secreted total VEGF protein in hypoxia. The *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ mRNA splice variants were all increased in hypoxic cells compared to normoxic controls. In normoxia, the relative *Vegf* splice variant mRNA levels ranked from highest to lowest for each cell type were *Vegf*₁₆₄>*Vegf*₁₂₀>*Vegf*₁₈₈. Hypoxic induction did not alter this ranking, although it did favor an increased stoichiometry of *Vegf*₁₆₄ mRNA over the other two splice variants. MC and astrocytes are likely to be the major sources of total *Vegf*, and *Vegf*₁₆₄ splice variant mRNAs, and VEGF protein in retinal hypoxia.

Keywords

Vascular endothelial growth factor; hypoxia; splice variants

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Introduction

Vascular endothelial growth factor (VEGF) is a potent endothelial cell (EC) mitogen, chemoattractant and regulator of vascular permeability (Risau 1997). VEGF drives physiologic vessel development, maintenance and maturation (Senger, Galli et al. 1983; Ferrara and Henzel 1989; Plouet, Schilling et al. 1989; Ferrara 2001) in fetal development as well as the adult, and is up-regulated in response to hypoxia, cytokines and other growth factors (Pertovaara, Kaipainen et al. 1994; Aiello, Northrup et al. 1995; Ben-Av, Crofford et al. 1995; Detmar, Brown et al. 1997; Ferrara and Davis-Smyth 1997; Jackson, Minton et al. 1997). Retinal and/or vitreal VEGF levels are elevated in neovascular retinal pathologies such as retinopathy of prematurity (ROP) (Lashkari, Hirose et al. 2000) proliferative diabetic retinopathy (PDR) (Aiello, Avery et al. 1994) and age-related macular degeneration (AMD) (Kvanta, Algvere et al. 1996; Lopez, Sippy et al. 1996). Furthermore, VEGF-directed therapies for the neovascular component of “wet” AMD have proven efficacious (Michels, Rosenfeld et al. 2005; Brown, Kaiser et al. 2006). Collectively, these observations indicate that VEGF is a primary inducer of pathological neovascularization.

In the human, alternative splicing of the *VEGF* gene leads to several distinct *VEGF-A* mRNA splice variants, namely, *VEGF*₁₂₁, *VEGF*₁₄₅, *VEGF*₁₄₈, *VEGF*₁₆₅, *VEGF*₁₈₃, *VEGF*₁₈₉ and *VEGF*₂₀₆. However, *VEGF*₁₂₁, *VEGF*₁₆₅, and *VEGF*₁₈₉ are generally considered the most abundant across the range of cell types that express *VEGF* (Zygalaki, Kaklamanis et al. 2008). The *VEGF* gene consists of 8 exons that are located on the 6th chromosome (Vincenti, Cassano et al. 1996). Exons 1–5 and 8 occur in all the known *VEGF* splice variants. Exons 6 and 7 encode heparin-binding domains and define structural and functional variations among the VEGF isoforms. *VEGF*₁₂₁ lacks exons 6 and 7 and it is freely diffusible (Ferrara, Houck et al. 1992; Shima, Kuroki et al. 1996). Splice variants with sequences derived from one or both of these domains tend toward increased matrix- and cell-association. *VEGF*₁₈₉ possesses partial sequence from exon 6 and the whole of exon 7; consequently, it is sequestered by ionic interactions with matrix- and cell surface-proteoglycans. *VEGF*₁₆₅ lacks exon 6 but incorporates exon 7 and has diffusion related- and cell-associated functions intermediate to those of *VEGF*₁₂₁ and *VEGF*₁₈₉. (Soker, Fidler et al. 1996; Ferrara and Davis-Smyth 1997; Neufeld, Cohen et al. 1999; Ng, Rohan et al. 2001; Robinson and Stringer 2001).

Angiogenesis is a process in which blood vessels sprout from a pre-existing vasculature, and vasculogenesis is the *de novo* blood vessel formation from EC precursors (Provis 2001). In humans, the development of the retinal superficial plexus begins at 16 weeks gestation; whether the retinal vasculature in humans develops by vasculogenesis, angiogenesis or a combination of both is controversial (Penn, Madan et al. 2008). The primary retinal vessel plexus originates from the optic disc and extends two-thirds the distance to the retinal periphery just beneath the retinal surface. Lateral and vertical angiogenic sprouts, originating from this advancing superficial plexus, extend the superficial network to the ora serrata while simultaneously generating the deep, or secondary, vascular plexus to the level of the inner nuclear layer. The overall result is an organized, bi-laminar retinal vascular network that is fully able to meet the metabolic demands of the inner retina (Stone, Itin et al. 1995; Hughes, Yang et al. 2000; Provis 2001) Retinal hypoxia resulting from the increased

metabolic demands of the developing neural retina, sometimes referred to as a physiologic hypoxia, induces VEGF expression and its regulation of normal retinal vasculature development.

Retinal hypoxia is a key feature and is common to the neovascular components of ROP, PDR and AMD. Hypoxia-induced retinal VEGF drives a neovascular response in each of these conditions leading to the development of disorganized, leaky blood vessels (Schaffer, Palmer et al. 1993; Wheatley, Dickinson et al. 2002). In ROP and PDR, these blood vessels penetrate the internal limiting membrane and enter the vitreous cavity, where they can lead to vitreous hemorrhage, retinal traction, detachment, and ultimately severe visual impairment (Foos 1985; Aiello, Avery et al. 1994). In AMD, neovascular structures arise from the choroid, penetrate Bruch's membrane, and enter the subretinal space where they can cause exudative detachment of the retina and death of photoreceptors (Geisen, McColm et al. 2006).

Müller cells (MC), astrocytes, retinal pigmented epithelial (RPE) cells, and retinal microvascular endothelial cells (RMEC) are known VEGF producers and, consequently, have the potential to contribute to the vasculogenic and/or angiogenic components of normal retinal vascular development and/or neovascular pathologies (Aiello, Northrup et al. 1995; Pierce, Avery et al. 1995; Stone, Itin et al. 1995; Miller, Adamis et al. 1997). There is little known regarding the cell-specific expression profiles and the specific functions of each VEGF isoform in the retina.

Amino acid sequence homology has made simultaneous identification and quantification of the individual VEGF isoforms impossible by traditional antibody-based methods. However, splice variant mRNA-specific detection and quantification has been performed by northern blot analysis, RNase protection assay and by quantitative real time RT-PCR (Klekamp, Jarzecka et al. 1999; Schwarzenbach, Chakrabarti et al. 2004; Mager, Renzetti et al. 2007). In this study, we have modified an existing real-time RT-PCR method to accurately quantify hypoxia-driven production of *Vegf* mRNA splice variants *Vegf*₁₂₀, *Vegf*₁₆₄, and *Vegf*₁₈₈ in four rat retinal cell types. These splice variants correspond to the human homologues *VEGF*₁₂₁, *VEGF*₁₆₅ and *VEGF*₁₈₉. Additionally, we have assayed total VEGF protein levels by ELISA and compared them to our *Vegf* mRNA expression data.

Materials and Methods

Cell Culture and Treatment

All retinal cell types were isolated from Long-Evans rats. Astrocytes were isolated as primary cultures from newborn (< 2 days old) rat pups. MC, RMEC and RPE were isolated from 2 to 10 day-old rat pups. Primary MC cultures were established from rat pups as previously described by Hicks and Courtois, and passage 1 through 3 MC were used for these experiments (Hicks and Courtois 1990). RPE cells were co-isolated with MC by an adaptation of the above method; primary and passage 1 RPE were used in these experiments. Retinal astrocytes were isolated using an immuno-magnetic separation technique (Su, Sorenson et al. 2003; McGinn, Poronnik et al. 2004) (Easy Sep™; StemCell Technologies) adapted from Sappington et al. (Sappington, Chan et al. 2006). PE-conjugated monoclonal

antibodies against astrocyte surface antigens [ab3268, ASTRO1 (10E4/R5), AbCam; Cambridge, MA] were used for astrocyte separation, and primary and passage 1 astrocytes were used for these experiments. RMEC were isolated using PECAM-1 (Becton Dickinson; Franklin Lakes, NJ) and a similar immuno-magnetic separation technique adapted from Matsubara et al. (Matsubara, Murata et al. 2000). Passage 1 and 2 RMEC were used in these experiments. The identity of MC isolates was confirmed by immunocytochemical staining with CRALBP (Abcam; Cambridge, MA); the identity of retinal astrocytes was confirmed by GFAP (Santa Cruz Biotechnology, Santa Cruz, CA); RMEC isolates were confirmed by VE-cadherin staining (Becton Dickinson; Franklin Lakes, NJ); and RPE cells were identified by morphology and the presence of cell pigmentation. The four retinal cell types are shown in figure 1. Following isolation, all cells were cultured at 37°C in a 5% CO₂/95% air atmosphere in a humidified incubator (NuAire; Plymouth, MN). Cultures were grown to 70% sub-confluence, and treated with serum-free medium, in normoxia or hypoxia, for 12 or 24 hours. Of note, our techniques required that the four cell types be isolated from retinas over a period of approximately one week between days <P2 and P10. This means that the cells may have come from retinal environments with different PO₂ levels, and this may have predisposed their responsivity to hypoxia in culture.

For treatment of cells with hypoxia, a CO₂-enriched environment was generated with a BBL™ GasPak Pouch system (Becton Dickinson; Franklin Lakes, NJ). The level of hypoxia obtained with these pouches is <0.7% O₂. The primary cultures and/or latter passages, for each cell type, showed no significant differences in the experimental endpoints.

Standard Curve Development

Total RNA was isolated and purified from cells using the Qiagen RNeasy Mini Kit (Qiagen; Valencia, CA). Contaminating genomic DNA was removed by RNase-Free DNase (Qiagen) treatment. RNA (0.5µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). PCR amplification of -RT controls was performed to test for any contaminating genomic DNA. Following cDNA synthesis, sequences specific for three *Vegf* splice variants, *Vegf*₁₂₀, *Vegf*₁₆₄, and *Vegf*₁₈₈, were amplified in a PCR reaction using primer sequences reported by Mager (Mager, Renzetti et al. 2007). These primers consist of one upstream primer, common to all three splice variants, and downstream primers that span splice junctions unique to each *Vegf* mRNA splice variant. Primers were synthesized by and purchased from Sigma (St. Louis, MO). The 50µl PCR reaction consisted of the following, template cDNA (3 µl of the RT reaction mixture), 1× PCR buffer (Applied Biosystems), 100 µM dNTP's (Applied Biosystems), up stream primer (0.5µM), down stream primer (0.5µM), Taq Polymerase (0.5µl) (Applied Biosystems) and RNase- free water. The cycle conditions are reported as follows: denaturation at 95°C for 10 minutes, 45 cycles of denaturation at 95°C for 0 sec, annealing at 55°C for 10 sec, extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 15 minutes.

Each *Vegf* splice variant-specific amplicon was electrophoresed on a 2% agarose gel to confirm the identity and purity. Each splice variant specific amplicon was excised from the gel, extracted using the QIAquick Gel Extraction Kit (Qiagen) and TA cloned using the

Invitrogen Topo TA 2.1 Cloning Kit (Invitrogen; Carlsbad, CA). Plasmids containing the splice variant-specific *Vegf* target sequences were extracted and purified from the bacterial colonies using the Qiagen Plasmid Mini Kit (Qiagen) and the inserts were sequenced to confirm identity. A 10-fold dilution series ranging from 10_9 to 10_3 copies per 3 μ l was prepared for each plasmid. PCR threshold cycles (Ct) vs. log of the known copy number were plotted for each dilution to prepare calibration curves for all the plasmids.

Splice Variant-Specific RT-PCR

Total RNA were isolated from cells using the All Prep DNA/RNA Mini Kit (Qiagen). RNA (0.5 μ g) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Following cDNA synthesis, splice variant-specific RT-PCR was performed in a 50 μ l reaction that consisted of iQ SYBR Green Supermix (BioRad; Hercules, CA), the common upstream VEGF primer (0.5 μ M), one of the splice-variant specific downstream primers (0.5 μ M), RNase free water and either standard or sample cDNA. cDNA samples from each treatment group, and the relevant dilution series described above, were co-amplified on the same microwell plate. Three biological replicates for each treatment group and three replicates for each dilution of the plasmid standards were used in these experiments. Each experiment was repeated at least three times.

ELISA

VEGF protein concentration was assayed in conditioned medium using a VEGF ELISA kit (R&D Systems; Minneapolis, MN). The amount of VEGF was normalized to total protein concentration cell lysates using a BCA assay (Pierce; Rockford, IL).

Data Analysis

VEGF splice variant-specific copy number was calculated by linear regression analysis using the relevant standard curve. The data are reported as copy number per ng of input RNA $\pm$ standard deviation, for each experimental group, for the four cell types. We chose to use an absolute qRT-PCR method and normalized our data to the mass of input RNA. In our view, this is the preferred method for measuring expression levels of mRNAs in hypoxia across varied retinal cell phenotypes. Normalization of target gene expression in relative qRT-PCR methods commonly employs the use of internal housekeeping genes such as β -actin (*ACTB*) and glyceraldehyde phosphate dehydrogenase (*GAPDH*). Although these mRNAs demonstrate stable expression in some experimental contexts, they are regulated by hypoxia, severely limiting their utility here (Zhong and Simons 1999). In our preliminary experiments, expression of both genes was significantly altered during hypoxic treatment of our cells. Furthermore, housekeeping gene mRNA levels are likely to change across the varied retinal cell types we investigated in this study. This variance could potentially add uncertainty to relative qRT-PCR expression data in addition to that associated with hypoxic regulation of a housekeeping gene used as a normalization control. The absolute method used here obviates the problems associated with relative expression to a hypoxia-regulated housekeeping gene. Secondly, it provides a reliable estimate of mRNA expression across different cell types and normal vs. cancerous surgical tissue specimens (Takeuchi, Bilchik et al. 2003; Aoyagi, Oda et al. 2004). To further validate our method, we normalized our copy

number data to total cell counts and found no significant changes compared to mass of input RNA normalization. We measured the steady-state *Vegf* mRNA and protein levels of retinal cells. Other studies, in other cell types, have shown that two regulatory events contribute to steady-state *Vegf* mRNA namely, hypoxia-induced *Vegf* gene transcription and mRNA stabilization (Forsythe, Jiang et al. 1996; Zhou, Gu et al. 2011). Furthermore, VEGF protein levels are determined by rates of translation and degradation. In this study, we did not perform the necessary experiments to determine the relative contributions of these regulatory processes.

Statistics were performed by ANOVA using the Stat View software version 5.0.1. (SAS Institute, Cary, NC). Differences having a p value < 0.05 were considered significant. Total *Vegf* mRNA copy # was estimated by summing the *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ copy numbers for experimental samples. No other splice variants were detected in any of the retinal cells tested.

Results

Müller Cell *Vegf* Splice Variant Production

MC cultured in normoxia vs. hypoxia expressed 99,847 vs. 347,381 total *Vegf* mRNA copy #/ng of input RNA, respectively, and these were the highest levels of expression compared to the other retinal cell types. Normoxic vs. hypoxic levels of the *Vegf* splice variants, reported as copy numbers/ng input RNA $\pm$ standard deviation, are shown in Figure 2. The values were: *Vegf*₁₂₀, $33,825 \pm 3,207$ vs. $92,218 \pm 10,150$ ($p=0.0007$); *Vegf*₁₆₄, $47,481 \pm 3,950$ vs. $195,174 \pm 22,642$ ($p=0.0004$); *Vegf*₁₈₈, $18,541 \pm 2,474$ vs. $59,989 \pm 8,847$ ($p=0.0014$). Hypoxic fold-induction for each splice variant in MC, expressed as the ratio of copy numbers/ng input RNA for hypoxia vs. normoxia, are reported as follows: *Vegf*₁₂₀, 2.70; *Vegf*₁₆₄, 4.11; and *Vegf*₁₈₈, 3.23. Compared to the other retinal cell types tested in this study, MC demonstrated the lowest hypoxic induction for each *Vegf* splice variant. The relative ratios of the *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ splice variant mRNAs in normoxia vs. hypoxia were 34 vs. 27%, 48 vs. 56% and 19 vs. 17%, respectively.

Astrocyte *Vegf* Splice Variant Production

In normoxia vs. hypoxia, astrocytes expressed 26,074 vs. 175,089 total *Vegf* mRNA copy #/ng input RNA, respectively, ranking second to MC under hypoxic conditions. However, the expression levels, under normoxic conditions, were lower than MC, higher than RMEC, and comparable to RPE. The *Vegf* splice variants, reported as copy numbers/ng of input RNA $\pm$ standard deviation, in normoxia vs. hypoxia, are shown in Figure 3. The values were: *Vegf*₁₂₀, $9,880 \pm 1,308$ vs. $47,319 \pm 9,128$ ($p=0.0022$); *Vegf*₁₆₄, $14,471 \pm 3,241$ vs. $120,849 \pm 27,232$ ($p=0.0026$); *Vegf*₁₈₈, 3.1 ± 0.6 vs. 12.7 ± 2.1 ($p=0.0009$). Astrocytes ranked second in terms of the hypoxic induction of *Vegf*₁₂₀ (4.79) and *Vegf*₁₆₄ (8.35), and third for *Vegf*₁₈₈ (4.01), compared to the other cells in this study. The relative ratios of the *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ splice variant mRNAs in normoxia vs. hypoxia were 38 vs. 27%, 55 vs. 69%, and 7 vs. 4% respectively.

RPE *Vegf* Splice Variant Production

In normoxia vs. hypoxia, RPE cells expressed 29,395 vs. 83,668 total VEGF mRNA copy #/ng input RNA, respectively, ranking 4th (lowest) under hypoxic conditions. In normoxia, RPE were comparable to astrocytes, ranking between MC (highest) and RMEC (lowest). Normoxic vs. hypoxic levels of the *Vegf* splice variants, reported as copy numbers/ng input RNA $\pm$ standard deviation, are shown in Figure 4. The values were: *Vegf*₁₂₀, 12,409 $\pm$ 2,335 vs. 50,183 $\pm$ 7,220 ($p=0.001$); *Vegf*₁₆₄, 14,471 $\pm$ 3,241 vs. 120,849 $\pm$ 27,232 ($p=0.0001$); *Vegf*₁₈₈, 4,484 $\pm$ 1,316 vs. 25,278 $\pm$ 3,962 ($p=0.001$). RPE cells ranked third for the hypoxic induction of *Vegf*₁₂₀ (4.0) and *Vegf*₁₆₄ (6.6), and second for *Vegf*₁₈₈ (5.6), compared to the other cells in this study. The relative ratios of *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ splice variant mRNAs in normoxia vs. hypoxia were 42 vs. 32 %, 43 vs. 52% and 15 vs. 16 % respectively.

RMEC *Vegf* Splice Variant Production

In normoxia vs. hypoxia, RMEC cells expressed 13,284 vs. 113,775 total *Vegf* mRNA copy #/ng input RNA, respectively, ranking 3rd under hypoxic conditions and 4th (lowest) in normoxia. Normoxic vs. hypoxic levels of the *Vegf* splice variants, reported as copy numbers/ng input RNA $\pm$ standard deviation, are shown in Figure 5. The values were: *Vegf*₁₂₀, 6,240 $\pm$ 1,567 vs. 31,074 $\pm$ 11,922 ($p=0.0232$); *Vegf*₁₆₄, 5,375 $\pm$ 1,035 vs. 72,087 $\pm$ 20,089 ($p=0.0046$); *Vegf*₁₈₈, 1,669 $\pm$ 176 vs. 10,614 $\pm$ 3,498 ($p=0.0115$). RMECs demonstrated the highest hypoxic induction of all the splice variants reported as follows: *Vegf*₁₂₀ (5.0), *Vegf*₁₆₄ (13.4) and *Vegf*₁₈₈ (6.4). The relative ratios of the *Vegf*₁₂₀, *Vegf*₁₆₄, and *Vegf*₁₈₈ splice variant mRNAs in normoxia vs. hypoxia were 46 vs. 27%, 41 vs. 63% and 13% vs. 9% respectively.

Total VEGF Protein Levels in the Conditioned Medium of Retinal Cells Cultured in Hypoxia or Normoxia

Secreted VEGF levels in conditioned medium of hypoxic vs. normoxic cultures are reported in pg/ml-mg $\pm$ SD and shown in Figure 6. Values were as follows: Müller cells, 1397.04 $\pm$ 227.07 vs. 25.12 $\pm$ 5.04, $p<0.0001$; astrocytes, 272.27 $\pm$ 64.27 vs. 114.04 $\pm$ 7.85, $p<0.0001$; RPE, 98.21 $\pm$ 5.73 vs. 62.59 $\pm$ 3.01, $p=0.0146$; and HRMEC, 9.97 $\pm$ 0.761 vs. 2.80 $\pm$ 0.334, $p=0.027$. These data indicate that hypoxic MC produced the highest secreted VEGF levels with a 56-fold increase over control normoxic cultures. Secreted VEGF levels of hypoxic astrocytes, RPE and RMEC ranked 2nd, 3rd and 4th; with hypoxic inductions of 2.4- 1.6- and -3.6 fold respectively.

Discussion

Knowledge of the structure/function relationships of the *VEGF* splice variants and their cell-specific expression profiles is critical to our understanding of the normal development of the retinal vasculature, as well as ocular neovascular diseases. Hypoxia is likely to be the primary stimulus for VEGF production by retinal cells in these settings (Pierce, Avery et al. 1995; Stone, Itin et al. 1995; Detmar, Brown et al. 1997; Miller, Adamis et al. 1997). Hence, we undertook this study to determine and compare the hypoxia-induced total VEGF protein and message, and *Vegf*₁₂₀, *Vegf*₁₆₄, *Vegf*₁₈₈ splice variants of four VEGF-producing retinal

cell types – MC, astrocytes, RPE cells and RMEC. It should be noted here that our total VEGF protein measurements did not account for the well-known cell-associated VEGF that can be present in VEGF-producing cells, perhaps causing us to underestimate the contribution of the heparin-binding forms under hypoxic conditions (Houck, Ferrara et al. 1991; Park, Keller et al. 1993). However the hypoxic induction profiles of VEGF total protein and *Vegf* mRNA were qualitatively similar and suggest that our total protein measurements in conditioned medium are a reliable measure of the cell-specific hypoxia-induced response at the protein level. MC expressed the highest secreted levels of VEGF by a very large margin, while astrocytes, RMEC and RPE ranked 2nd, 3rd and 4th, respectively. The VEGF protein and total *Vegf* mRNA levels for MC and astrocytes in hypoxia, showed similar trends. *Vegf* mRNA levels in hypoxic RPE and RMEC were comparable, yet the corresponding VEGF total protein levels were significantly higher for RPE. In each cell type, the *Vegf*₁₆₄ splice variant mRNA was the most abundant in hypoxia, and hypoxic exposure favored an increased *Vegf*₁₆₄ population within the total *Vegf* mRNA pool.

In whole body knockout mice, sole expression of any of the *Vegf*₁₂₀, *Vegf*₁₆₄ or *Vegf*₁₈₈ variants, exclusive of the other two, resulted in three strains with varied retinal vascular phenotypes. Retinal vascular development was normal in *Vegf*^{164/164} mice, yet defective in the *Vegf*^{120/120} and *Vegf*^{188/188} strains (Stalmans, Ng et al. 2002). Rat retinal *Vegf*₁₆₄ mRNA was significantly higher than *Vegf*₁₂₀ and *Vegf*₁₈₈ mRNAs on postnatal day 7 (p7), when the retinal vasculature had reached mid-periphery, and it remained elevated through postnatal day 18 (p18). *Vegf*₁₂₀ levels decreased by p18 while *Vegf*₁₈₈ remained at low levels that were not significantly different from p7 through p18 (McColm, Geisen et al. 2004). We found that astrocytes isolated from rats during retinal development and exposed to hypoxia expressed lower levels of *Vegf*₁₈₈ mRNA compared to *Vegf*₁₆₄ mRNA. Furthermore, they demonstrated a higher hypoxic induction of *Vegf*₁₆₄ relative to *Vegf*₁₈₈. It has been hypothesized that astrocytes guide the vasculogenic and/or angiogenic component(s) of normal retinal vascular development, as they reside in close proximity to developing vessels of the primary plexus, migrating ahead of the vascular bed and secreting a trail of VEGF in response to tissue hypoxia (Stone, Itin et al. 1995). It could also be hypothesized that a preponderance of the matrix- and cell surface-bound VEGF₁₈₈ is secreted during this process, providing directionality to the developing vascular plexus. Our findings indicate that there is no hypoxia-induced shift in stoichiometry favoring the *Vegf*₁₈₈ splice variant mRNA and, when considered together with reports from other laboratories, this suggests that astrocyte-derived VEGF₁₆₄ plays a dominant role over the other two isoforms in the retinal vascular development of rodents. However, we did not assay the VEGF isoforms, nor have there been any other reports that describe these data. If indeed there is a shift toward higher levels of the VEGF₁₈₈ isoform in this context, then other regulatory pathways, stimuli and/or protein factors may be involved. Conversely, retinal vascular development in mice with a conditional astrocyte-specific total *Vegf* genetic deletion was normal challenging the notion that hypoxia-induced astrocyte-derived VEGF is important to this process (Weidemann, Krohne et al. 2010). However these data are inconclusive because it is plausible that retinal ganglia, macrophages and/or microglia may assume the role of VEGF producers, normally performed by astrocytes.

A pathologic pre-retinal neovascularization (NV) occurs in rodent oxygen-induced retinopathy (OIR), and in the human populations suffering from ROP and DR (Penn, Madan et al. 2008). RMEC of the superficial retinal vascular plexus respond to VEGF derived from hypoxic retinal cells by angiogenic sprouting through the inner limiting membrane, extending into the vitreous cavity. Experimental evidence suggests that MC and astrocyte-derived VEGF contribute significantly to OIR-induced NV. For example, in mouse OIR, a conditional astrocyte- or MC-specific genetic deletion of *Vegf* reduced pre-retinal neovascularization by 46 and 40% respectively (Bai, Ma et al. 2009; Weidemann, Krohne et al. 2010). In a previous study, immunohistochemical and *in situ* hybridization analyses of retinal tissue indicated that MC are likely to constitute the main source of VEGF in OIR rats with pre-retinal NV (Robbins, Conaway et al. 1997). Our *in vitro* data indicate that *Vegf*₁₆₄ is the most abundant splice variant expressed by MC and astrocytes in hypoxia, suggesting its central importance in the hypoxia-induced neovascular response of the superficial vascular plexus in rodents and humans.

Leukostasis is a component of retinal inflammation and may contribute to pre-retinal NV in DR (Huang, Gandhi et al. 2011; Tang and Kern 2011; Zhang, Liu et al. 2011). Leukostasis leads to retinal capillary occlusion and subsequent vasoregression; RMEC downstream from the occlusion become hypoxic and consequently may elaborate VEGF and other cytokines contributing to the onset of NV. Similarly, subretinal neovascularization is a blinding component of AMD, and one theory of its pathogenesis describes an age-dependent vasoregression of the choriocapillaris with incipient hypoxia experienced by the RPE (Kurihara, Westenskow et al. 2012). Accordingly, the RPE respond by increasing hypoxia-dependent expression and secretion of VEGF, which may induce a neovascular response from the choroid. Our *in vitro* studies of the hypoxic induction of RPE and RMEC are relevant to these *in vivo* settings, and the stoichiometry of hypoxia-induced *Vegf* splice variant expression we observed, again, points to a critical and perhaps dominating role for *Vegf*₁₆₄.

Most significantly, our findings indicate that Müller cell- and astrocyte-derived VEGF protein levels induced by hypoxia are dramatically higher than RPE and RMEC. Hypoxia induces RPE to express and secrete VEGF *in vitro*, suggesting that the RPE is a paracrine source of VEGF soliciting a choroidal neovascular response in AMD. Our studies also reveal that very little VEGF is expressed and secreted by RMEC compared to the other retinal cell types, suggesting that they are not a paracrine source. Furthermore, our data provide some insight into the relative contributions of hypoxia-induced retinal cells to *Vegf* splice variant mRNA expression. In the four cell types investigated in this study, *Vegf*₁₆₄ mRNA was expressed at the highest levels in normoxia and hypoxia, and hypoxic induction increased the expression of all the splice variants while trending toward a stoichiometry in favor of *Vegf*₁₆₄. Our data suggests that *hypoxia alone* favors increased *Vegf*₁₆₄ expression. Therefore, if increased production of the other VEGF isoforms is required to satisfy a specific physiologic or pathophysiologic demand, other regulatory pathways, protein factors and/or stimuli may be required.

Future studies should be directed at defining the precise role of each cell type in normal vascularization and pathologic neovascularization in the retina.

Acknowledgments

Supported by the Knights Templar Eye Foundation (DGM), the International Retinal Research Foundation (DGM), NIH EY07533 (JSP) and an Unrestricted Grant from Research to Prevent Blindness, Inc.

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Highlights

- *Vegf*₁₂₀, *Vegf*₁₆₄ and *Vegf*₁₈₈ are three VEGF splice variants expressed in the rat retina.
- Splice variant-specific expression of VEGF in four retinal cell types was assayed in response to hypoxia.
- Total VEGF protein in the conditioned medium of hypoxic and normoxic cells was assayed.
- *Vegf*₁₆₄ was the most abundant splice variant in all four retinal cell types.
- Of the four cell types, Müller cells produced the highest total *Vegf* mRNA and protein levels.

Figure 1.
Immunocytochemical staining of rat retinal cells: **A)** Müller cells with anti-CRALBP; **B)** astrocytes with anti-GFAP; **C)** retinal pigmented epithelium (unstained); **D)** microvascular endothelial cells with anti-VE-cadherin.

Muller Cells

Figure 2.

Muller cells were cultured in hypoxia or normoxia for 12 hours. The cells were lysed. Total RNA was isolated, reverse transcribed, and analyzed by a validated, splice variant specific, external calibration curve qRT-PCR method. The expression levels are reported as *Vegf* copy numbers/ng input RNA $\pm$ standard deviation.

Astrocytes

Figure 3.

Astrocytes were cultured in hypoxia or normoxia for 12 hours. The cells were lysed. Total RNA was isolated, reverse transcribed, and analyzed by a validated, splice variant specific, external calibration curve qRT-PCR method. The expression levels are reported as *Vegf* copy numbers/ng input RNA $\pm$ standard deviation.

RPE Cells

Figure 4.

Retinal pigment epithelial cells were cultured in hypoxia or normoxia for 12 hours. The cells were lysed. Total RNA was isolated, reverse transcribed, and analyzed by a validated, splice variant specific, external calibration curve qRT-PCR method. The expression levels are reported as *Vegf* copy numbers/ng input RNA $\pm$ standard deviation.

RMEC

Figure 5. Retinal microvascular endothelial cells were cultured in hypoxia or normoxia for 12 hours. The cells were lysed. Total RNA was isolated, reverse transcribed, and analyzed by a validated, splice variant specific, external calibration curve qRT-PCR method. The expression levels are reported as *Vegf* copy numbers/ng input RNA $\pm$ standard deviation.

Figure 6.

Müller cells, retinal microvascular endothelial cells, retinal pigmented epithelium cells and astrocytes were cultured in hypoxia or normoxia for 24 hours. Conditioned media were collected and assayed for total VEGF by ELISA. The VEGF levels were normalized to cellular protein.