Reduction in endothelial tip cell filopodia corresponds to reduced intravitreous but not intraretinal vascularization in a model of ROP

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Abstract

Purpose—To determine the effect of a vascular endothelial growth factor receptor 2 tyrosine kinase (VEGFR2) inhibitor on intravitreous neovascularization (IVNV), endothelial tip cell filopodia, and intraretinal vascularization in a rat model of retinopathy of prematurity (ROP).

Methods—Within 4 hours of birth, newborn Sprague-Dawley rats pups and their mothers were cycled between 50% and 10% oxygen daily until postnatal day (p)12. Pups were given intravitreous injections of VEGFR2 inhibitor, SU5416, or control (dimethyl sulfoxide, DMSO) and returned to oxygen cycling until p14, then placed into room air. Intravitreous neovascularization (IVNV), avascular/total retinal areas, and endothelial tip cell filopodial number and length were determined in lectin-labeled neurosensory retinal flat mounts. Cryosections or fresh tissue were analyzed for phospho-VEGFR1, phospho-VEGFR2, activated caspase 3, or phospho-β3 integrin,. Human umbilical venous (HUVECs) and human choroidal endothelial cells (ECs) were treated with VEGFR2 inhibitor to determine effect on VEGFR2 phosphorylation and on directed EC migration toward a VEGF gradient. Filopodial length and number of migrated ECs were also measured.

Results—Compared to control, the VEGFR2 inhibitor reduced VEGFR2 phosphorylation in HUVECs in vitro and clock hours and areas of IVNV but not percent avascular retina in vivo. Filopodial length and number of filopodia /EC tip cell were reduced in retinal flat mounts at doses that inhibited IVNV, whereas at lower doses, only a reduction in filopodial length/ EC tip cell was found. There was no difference in phosphorylated β3 integrin and cleaved caspase-3 labeling in VEGFR2 inhibitor-treated compared to control in vivo. Doses of the VEGFR2 inhibitor that reduced filopodial length and number of filopodia/migrating EC corresponded to reduced EC migration in in vitro models.

Conclusions—VEGFR2 inhibitor reduced IVNV and filopodial number and length/EC tip cell without interfering with intraretinal vascularization. Reducing the number and length of filopodia/ endothelial tip cell may reduce guidance cues for endothelial cells to migrate into the vitreous without interfering with migration into the retina toward a VEGF gradient.

Keywords

vascular endothelial growth factor (VEGF); retinopathy of prematurity (ROP); intravitreous neovascularization; intraretinal neovascularization; SU5416; filopodia

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INTRODUCTION

Signaling through vascular endothelial growth factor (VEGF) and its receptors is recognized as important in the development of intravitreous neovascularization in retinopathy of prematurity (ROP), a leading cause of childhood blindness world-wide (Chen J and Smith LE 2007). Reports indicate that intravitreous agents that inhibit the bioactivity of VEGF can successfully reduce the vascular activity in some cases of severe ROP (Chung E.J. et al. 2007;Travassos A. et al. 2007). However, several issues remain including the timing of treatment, dose, and mechanism of reducing VEGF activity. Using an animal model of ROP, we found that the dose of a neutralizing antibody to VEGF was critical. Too low a dose appeared to result in an increase in clock hours of intravitreous neovascularization (IVNV) and in VEGF protein within the retina (Geisen et al. 2008). Since it is not feasible to measure VEGF concentration in the individual human preterm infant retina, determination of a safe and effective dose of antibody may not be possible currently. Furthermore, there are potential safety concerns of effects of anti-VEGF agents on the retina and on other organs from absorption into the bloodstream of the developing infant. The timing of dose is important as well. Intravitreous bevacizumab has been reported to hasten fibrous contraction to cause a total retinal detachment in an infant with ROP(Honda S. et al. 2008). Therefore, other treatment strategies are needed.

Besides the role VEGF plays in pathologic IVNV, it also provides endothelial and neuronal survival cues (Oosthuyse et al. 2001;Nishijima et al. 2007) and is essential for normal retinal vascular development (Carmeliet et al. 1996;Chan-Ling et al. 1995;Stone et al. 1995;Ferrara 2001), which is ongoing in the premature infant. Stimulation of VEGF receptor 1 (VEGFR1) with either VEGFA or placental growth factor prior to the hyperoxia induced vaso-obliterative phase of oxygen-induced retinopathy protected against pathologic neovascularization (Shih et al. 2003). In addition, a slow release antibody to VEGFR2, the receptor involved with most angiogenic processes (Rahimi 2006), reduced IVNV in a dog model of ROP. However, retinal vascular development was delayed in both the treated and control groups compared to room air raised pups (McLeod et al. 2002), raising the question whether inhibition of VEGFR2 signaling affected ongoing retinal vascularization. We were interested in the effects of short term inhibition of VEGFR2 signaling on IVNV and ongoing vascular development. To study this, we used a receptor tyrosine kinase inhibitor to VEGFR2 in a relevant model of ROP, the rat 50/10 OIR model (Penn et al. 1994).

MATERIALS AND METHODS

All animal studies complied with the University of North Carolina's Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Model of Oxygen Induced Retinopathy (50/10 OIR Model)

Litters of 12-16 newborn Sprague-Dawley rat pups (postnatal age 0= p0) with their mothers (Charles River, Wilmington, MA) were placed into an Oxycycler incubator (Biospherix, New York, NY), which cycled oxygen between 50% O_2 and 10% O_2 every 24 hours until p14, at which time pups were returned to room air for 4 or 11 days(Penn, Henry, and Tolman 1994). Oxygen levels were monitored and maintained within $\pm$ 0.5% and carbon dioxide in the cage was monitored and flushed from the system by maintaining sufficient gas-flow. The model developed IVNV at p18(Werdich and Penn 2006), similar to acute Stage 3 ROP. The 50/10 OIR model also undergoes natural regression of IVNV with intraretinal vascularization toward the ora serrata(Penn et al. 1994; Hartnett et al. 2006; Geisen et al. 2008).

Intravitreous Injections

At p12, rat pups were anesthetized with an intraperitoneal (IP) injection of a mixture of ketamine (20 mg/kg) and xylazine (6 mg/kg) (both from NLS Animal Health, Pittsburgh PA). A topical anesthetic (0.5% tetracaine hydrochloride) was administered prior to inserting a 30-gauge needle just posterior to the limbus to avoid lens damage. One μL injections were performed in one eye using a UMP3 Nanofill Injection System (WPI Inc., Sarasota, Fl) and all fellow eyes were not injected. Topical antibiotic ointment (0.5% erythromycin, Fougera, Melville NY) was applied after injections. Animals were monitored until recovery (~2 hours) and then returned with their mothers to the Oxycycler for two more days. Pup body weights were measured at the time of intervention and only those litters with mean body weight ± 2 g of one another were used in experiments, because body weight can affect outcomes (Holmes and Duffner 1996).

Inhibiting VEGF Bioactivity

SU5416 (EMD Biosciences, San Diego, CA) is a synthetic, non-peptide, receptor tyrosine kinase inhibitor of VEGFR2 that has been well-characterized (Fong et al. 1999). The compound was dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh) and then diluted with PBS to result in the following concentrations that were delivered in 1 μL intravitreous injections: 33 mg/ml (200 uM), 16.5 mg/ml (100 uM), 8.3mg/ml (50 uM). DMSO (0. 5%) was used as a control.

Fresh Tissue Preparation

Animals were euthanized with pentobarbital (80 mg/kg IP). Both eyes were enucleated and the retinas were isolated under a dissecting microscope using a modification of the method of Chan-Ling (Chan-Ling 1997), in which anterior segments were removed and the retinas without ora serratas were dissected free from remaining sclera, choroid, hyaloidal vessels and vitreous. The tissue was placed in radioimmuno precipitation assay (RIPA, .5% sodium deoxycholic acid, 20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1% Triton x-100, 0.1% SDS, 10% Glycerol) with a protease inhibitor cocktail (1:100) and 1 M orthovandate (1:100, Sigma, St. Louis, MO) and frozen at −20°C for later analyses of protein by western blot and ELISA.

ELISA for VEGF

Supernatants from frozen protein samples prepared with RIPA and protease inhibitors were centrifuged at 16,000g. Samples were assayed without dilution in duplicate using commercially available ELISA kits for rat $VEGF_{164}$ isoform (R & D Systems, Minneapolis).

Tissue Culture of HUVECs

Human umbilical vein endothelial cells (HUVECs) (Cambrex, Walkersville MD) express VEGF receptors (Thieme et al. 1995) and were used up to passage 7. HUVECs grown to 70% confluency (~ 3 days) in EGM-2 with 2% FBS (Cambrex, Walkersville, MD), were starved overnight in EBM-2 (without supplied growth factors) with 0.5% FBS, and then incubated with SU5416 or 0.5% DMSO for 2 hours before stimulation with VEGF 50ng/ml for 30 minutes. Cells were immediately lysed in RIPA buffer containing protease inhibitor cocktail (Sigma, St. Louis, MO) and 2 μm orthovandate (Sigma, St. Louis MO).

Western Blot Analysis

Total VEGFR1 and VEGFR2 were immunoprecipitated at a concentration of 1μg of antibody per 100 μg of protein sample. Equal amounts of protein samples were resolved by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to PVDF membranes (Bio-Rad, Hercules, CA), which were blocked with 5%BSA/TBS or 5% Blocking

grade dry milk (catalog # 170-6404, Biorad) for 1 hour prior to incubation with the primary antibody. Membranes were probed with anti-phospho-VEGFR1 (Upstate, Lake Placid, NY), anti-phospho-VEGFR 2 (Cell Signaling, Danvers, MA), anti-VEGFR1 or anti-VEGFR2 (Santa Cruz Biotech, Santa Cruz, CA) at a dilution of 1:200. The membranes were then incubated with horseradish peroxidase (HRP)—conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) and visualized by enhanced chemiluminescence (PIERCE, IL). Digitized images of Western blots were quantified using UN-SCAN-IT (Silk Scientific, Orem, Utah). The densitometric values of phospho- VEGFR1 and phospho-VEGFR2 were normalized against those of the total VEGFR1, and total VEGFR2 respectively.

Dissecting Retinal Tissue for Flat mounting and Cryosections

At p14 or p18, pups were anesthetized by intraperitoneal (IP) injection of ketamine (60 mg/ kg) and xylazine (18 mg/kg). Paraformaldehyde (PFA)(1.0 mL, 0.5%) was directly perfused into the left ventricle prior to euthanasia by intracardiac injection of pentobarbital (50ul, 80mg/ kg). Both eyes were enucleated and fixed in 2% paraformaldehyde (PFA) for 2 hours. Retinas were isolated as described for fresh tissue except that ora serratas were kept intact. By making 4 incisions 90 degrees apart, the retinas were flattened and then placed onto microscope slides. For cryosections, intact fixed eyes with only the cornea, lens and vitreous removed, were put into 30% sucrose/PBS overnight. Each eye was blotted with filter paper to remove excess liquid, soaked in Optimal Cutting Temperature compound (O.C.T., Tissue-Tek, Torrance, CA) and kept at −80 °C for future analysis.

Tissue Staining

The flattened retinas were permeabilized in ice cold 70% v/v ethanol for 20 minutes, then in PBS/1% Triton x-100 for 30 minutes, and then incubated with Alexa Fluor 568 conjugated G. simplicifolia (Bandeiraea) isolectin B4 (5 µg/ml, Molecular Probes, OR) in PBS overnight at 4°C for staining of the vasculature. The slides were rinsed three times in PBS and mounted in PBS:glycerol (2:1) with VectaShield (Vector Labs, CA), and the cover slip sealed with nail varnish. Images of the retinal blood vessels were captured using a Nikon TE2000U inverted microscope (Michael-Hooker Microscopy Facility, University of North Carolina, Chapel Hill) and digitally stored for analysis. Image sections were assembled using methods that maintained the original image dimensions and that did not induce image distortion using Tekmate's PhotoFit Premium v1.44 or with Adobe Photoshop 7.0.

Tissue Staining for Cryosections

Eyes frozen in O.C.T. were cut into 10-µm sections, adjacent to or within 10 µms of one another. For qualitative comparisons, labeled serial sections were all placed on the same microscope slide to assure equal handling and antibody labeling conditions. Sections were first incubated in PBS/1% Triton X-100 for 30 minutes. Some retinas were incubated with Alexa Fluor 568- isolection B4 in PBS for 30 minutes at room temperature to stain the vasculature. After washing in PBS three times, retinas were incubated for 30 minutes in 3% normal goat serum or 5% BSA to block nonspecific binding of the primary antibody. Anti-phospho-integrin β3 (Santa Cruz, Santa Cruz, CA), a polyclonal rabbit antibody, was used at a dilution of 1:200. Anti-caspase-3 (Cell Signaling, Danvers, MA) was used at a dilution of 1:50. Retinas were incubated overnight at 4 °C. After three washes in PBS, retinas were incubated for one hour with a 1:500 dilution of goat anti-rabbit conjugated with Alexa-488 (Invitrogen, Carlsbad, CA). All retinal cryosections were rinsed three times in PBS, then some were incubated with a 1:5,000 dilution of Hoechst 33342 (Invitrogen, Carlsbad, CA) for 15 minutes, and all were mounted in PBS-glycerol (2:1 with VectaShield; Vector Laboratories, Burlingame, CA). On all slides, the cover slips were sealed with nail polish and images of the sections were captured with a scanning laser confocal microscope (Leica SP2, Wetzlar, Germany) and digitally stored for analysis.

Control sections were labeled with secondary antibodies to control for background staining due to non-specific binding.

Measurement of Intravitreous Neovascularization (IVNV)

To determine the extent of IVNV, retinal images from injected and fellow non-injected eyes from experimental and control groups were randomized, labeled, and analyzed for IVNV clock hours and percent IVNV area to total retinal area. Two masked reviewers performed all analyses. The presence of IVNV was determined with a technique adapted from those used in clinical trials(Early Treatment for Retinopathy of Prematurity Cooperative Group 2003) and animal model determination(Zhang et al. 2000). For clock hours, flat mounts were divided into 12 clock hours of approximately equal area using Adobe Photoshop, assessed for the presence of IVNV(Zhang, Leske, and Holmes2000;Early Treatment for Retinopathy of Prematurity Cooperative Group2003), and assigned a number (0 to 12) based on the number of clock hours exhibiting IVNV. IVNV area was quantified using ImageTool (University of Texas Health Science Center, San Antonio). The area in each clock hour exhibiting IVNV was measured and added together as the total IVNV area per retina and expressed as a percent of the total area of the retina.

Analysis of Peripheral Avascular Areas and Quantification of Capillary Density

Digitized images of the total retinal area and peripheral avascular areas were measured (ImageTool v.3, University of Texas, TX). The peripheral avascular area was expressed as a percentage of the total retinal area for experimental and control eyes.

In Vitro Horizontal Directed Endothelial Cell Migration toward a VEGF Gradient Assay

Choroidal endothelial cells (ECs) were plated in a 3D matrigel layer on Nunc 4 well chamber slides. A matrigel "spot" with 30 ng/ml of VEGF was first added to the center of each chamber and allowed to polymerize at 37 °C. A second matrigel layer with 3 ng/ml of VEGF and 3×10^4 cells stained with Vybrant DiO was oriented around the first. Both layers were also treated with 2, 5, or 10 μM of the VEGFR2 inhibitor or DMSO control. ECs were allowed to proliferate overnight at 37 °C with 5% CO_2 in EGM-2 media with 10% FBS and treated with the same concentrations of the VEGFR2 inhibitor or DMSO. After 24 hours, the number of cells entering the first layer was counted with a light microscope. Images of individual cells were taken with a Leica confocal microscope. For each well, ten cells that entered the first Matrigel layer were used for analysis and three wells/condition were averaged. The filopodia were counted and lengths were measured with ImageTool. Only filopodia that measured at least 10 microns in length and less than 0.75 microns in diameter were analyzed.

Modified Boyden EC Migration Assay (Vertical EC Assay)—A thin layer of Matrigel containing recombinant $VEGF_{165}$ (50 ng/ml, R&D Systems, MN), was applied to 24-well plates. 500 μl of serum free endothelial basal media (EBM-2; Lonza, Switzerland) was added to each well followed by a 6.5 mm diameter Transwell insert (8 μm pores; Corning, NY). Isolated human choroidal endothelial cells (ECs, passage 2) in EBM-2 were prestained with Vybrant DiO (Invitrogen, OR) for 30 minutes at 37 °C and seeded into the inserts at 50,000 cells per 200 μl of serum free EBM-2 media. All inserts and wells were treated with either 0, 2, 5, or 10 μM SU5416 in .5% DMSO. The untreated control contained no $VEGF_{165}$.

The plates were allowed to incubate for 16 hours at 37 °C, 5% CO_2. At the endpoint, the ECs inside the insert (unmigrated) were removed with a moist Q-tip. The migrated ECs attached to the underside were left intact. The ECs were imaged with an Olympus CK40 microscope and Olympus DP71 camera (PA). Multiple images of five different areas were taken from each insert. ECs in every area were counted and an average cell number for each insert was obtained.

There were 5 wells/treatment condition for each experiment and this was repeated 3 times. Results were analyzed with ANOVA using Bonferroni correction.

Statistical Analysis

Means ± standard errors were reported. In cases with more than 2 groups analyzed, an overall analysis of variance (ANOVA) was performed with post-hoc Bonferroni correction. When the question required comparison of only two groups, Student t-tests were used. Each group compared in any analysis had at least 5 or more pups from at least 2 litters. For all comparisons, an alpha level of <0.05 was used as criterion of significance.

RESULTS

VEGFR2 inhibitor reduces VEGFR2 phosphorylation in HUVECs

To determine a dose for in vivo inhibition of angiogenesis, we exposed cultured HUVECs to different concentrations of VEGFR2 inhibitor, SU5416, before addition of 50 ng/ml VEGF protein. There was a significant reduction in VEGFR2 phosphorylation (Figure 1A) compared to DMSO control. However, VEGFR1 phosphorylation was unaffected at the same doses (Figure 1B). Our findings did not support other studies that reported that SU5416 inhibits VEGFR1 phosphorylation and c-kit in some systems (Smolich et al. 1992;Kosmider et al. 2006).

VEGFR2 inhibitor Reduces VEGFR2 phosphorylation and IVNV

Retinas from rats in the 50/10 OIR model were measured for VEGF protein at p12, p13, p14 and p18. Of the 4 time points, VEGF protein was greatest at p13 during hyperoxia and following exposure to 10% O_2 after 6 complete fluctuations in oxygen cycling (Figure 2). We therefore chose p12 to perform intravitreous injections and used several doses of the VEGFR2 inhibitor based on the in vitro experiments and estimated dilution effect of the vitreous volume (Berkowitz et al. 1998). Since there were no differences in clock hours of IVNV among DMSO controls, we combined all controls into one group. At p18, the time point of maximal clock hours of IVNV, there was a dose-dependent reduction in clock hours and area of IVNV (Figure 3a and 3b). Compared to control there was approximately a 60% reduction in IVNV area and clock hours with the 200 μM injection of SU5416 (p≤0.02; Figure 3). At p13, using the same dose, there was reduced phosphorylation of VEGFR2 (Figure 4a), but not VEGFR1 (Figure 4b), determined by immunoprecipitation and western blot.

We previously found that a dose of neutralizing antibody to VEGF, effective at inhibiting IVNV, led to an increase in retinal VEGF one day following treatment (Geisen et al. 2008). We determined the VEGF concentration in retinas from pups exposed to the 50/10 OIR model that had received the 200 μM injection of VEGFR2 inhibitor or DMSO at p12 and were analyzed at p13 and p14 (Figure 5). There was no difference in VEGF protein by ELISA between DMSO or SU5416 at either time point, although consistent with Figure 2, VEGF was greater at p13 than at p14 in control and treated groups.

Filopodial extension from endothelial tip cells in 50/10 OIR

Since VEGF gradients have been found important in filopodial extension, direction of endothelial tip cells, and have been proposed to be important in endothelial migration(Gerhardt et al. 2003; Bentley et al. 2008), we determined whether inhibition of VEGFR2 signaling reduced the number or length of filopodia on endothelial tip cells in the 50/10 OIR model (Figure 6). In the VEGFR2 inhibitor group, we found a significant reduction in total length of filopodia/endothelial tip cell (ANOVA p<0.001, post-hoc tests between the DMSO control and 10 μM (p=0.001) and between 10 μM and 200 μM (p=0.005), Figure 6b), but only the 200

μM injection of SU5416 significantly reduced number of filopodia per tip cell compared to control (ANOVA, p<0.001; post-hoc protected t-test between DMSO and 200 μM injection of SU5416, p=0.001, Figure 6c).

Effects of inhibiting VEGFR2 on Avascular Retinal Area

Since inhibition of VEGFR2 signaling reduced filopodial length, we reasoned that it would not only reduce clock hour extent or area of IVNV, but might also prevent ongoing retinal vascular development, which would be an adverse event in ROP. The 50/10 OIR model is particularly useful to study this, because it causes arterial oxygen concentrations in the rat (Penn et. al 1994) similar to the transcutaneous oxygen extremes experienced in human infants that develop severe ROP(Cunningham *et al.* 1995), exposes pups to fluctuations in oxygen, a risk factor for severe ROP(Hong *et al.* 2002;Cunningham, et al.1995;Chow *et al.* 2003), and reproducibly and consistently develops IVNV and avascular retina similar in appearance to acute Stage 3 ROP(Penn et al.1994;Hartnett, et al.,2006). Pups exposed to the 50/10 OIR model and treated with the VEGFR2 inhibitor had no difference in avascular/total retinal area compared to control at p18 (Figure 7). We found no difference in staining for cleaved caspase-3 in cryosections from eyes treated with 200 μM SU5416 injection compared to control (data not shown).

β3 integrin phosphorylation

β3 integrin is involved in VEGFR2 signaling and its phosphorylation can activate pathways leading to endothelial migration(Borges et al. 2000). To determine whether phosphorylation of β3 integrin was affected by SU5416, we stained cryosections from 50/10 OIR model at p13 and p14 that had been treated with VEGFR2 inhibitor with an antibody to phosphorylated β3 integrin. In control and in the 10 or 200 μM VEGFR2 inhibitor treated eyes there was staining for phosphorylation of β3 integrin in the inner capillary plexus. Compared to DMSO, phosphorylation of β3 integrin did not appear to be qualitatively affected by inhibition of VEGFR2 with SU5416 (Figure 8).

In vitro studies

We found that inhibition of VEGFR2 signaling reduced filopodial length and number and IVNV, but did not appear to adversely affect intraretinal vascularization that naturally occurs in the 50/10 OIR model. To model intraretinal vascularization, we developed an in vitro assay to assess horizontal directed EC migration toward a VEGF gradient. Within 24 hours of plating choroidal ECs, there was directed migration with filopodia extending toward the VEGF gradient within the central matrigel pad (Figure 9a). The VEGFR2 inhibitor caused a decrease in filopodia length/endothelial cell (ANOVA, p=0.03, Figure 9b) but did not affect the number filopodia/ EC (Figure 9c) or the number of migrating ECs (data not shown). However, EC migration toward a vertical VEGF gradient (Figure 9d) across a porous insert was reduced in a dose-dependent fashion (Figure 9e).

DISCUSSION

Using a rat model of ROP, we showed that inhibiting the receptor tyrosine kinase of VEGFR2 using SU5416(Ahmed et al. 2004) significantly reduced intravitreous neovascularization. Since VEGF is important in angiogenesis, we anticipated that inhibition of signaling through its main angiogenic receptor might reduce both intravitreous and intraretinal neovascularization. However, we found no interference with ongoing intraretinal vascularization that naturally occurs in the 50/10 OIR model (Geisen, et al. 2008).

The VEGFR2 inhibitor, SU5416, is a small non-peptide synthetic molecule that is cleared within several days(Ye et al. 2006) and VEGFR2 phosphorylation was already restored at p14.

SU5416 also did not have an effect on retinal VEGF at p13 or p14. Although retinal VEGF is reduced at p18 compared to p13 in the 50/10 OIR model, retinal VEGF is 10 fold greater than that in the vitreous(Geisen, et al. 2008). One reason why intraretinal vascularization may have proceeded was that the gradient of VEGF was still in favor of intraretinal rather than intravitreous neovascularization once the inhibition of the receptor signaling wore off. However, that reasoning alone does not explain why the angiogenesis proceeded into the vitreous initially.

The VEGF gradient is important in endothelial tip cell filopodial direction and extend both in normal development and when excessive VEGF is in the vitreous (Gerhardt et al., 2003). Although several investigators have reported that filopodial extension occurs in directed endothelial migration toward a gradient (Pi X *et al*. 2007;Levchenko *et al*. 2008), it remains unclear whether filopodial extension or number is essential for endothelial migration in vivo. For example, in neurobiology, filopodial extension though important in determining guidance cues for neuronal extension, was not found to be necessary for axonal elongation (Bentley and Toroian-Raymond 1986). Therefore, decreasing the length and number of filopodia may effectively reduce guidance cues for endothelial cells to migrate into the vitreous without affecting likely more proximal ones to migrate into the retina. In artificial systems, fibroblast filopodia can extend across regions where adherence to matrix is not present to islands where adherence can occur (Guillou *et al*. 2008). Filopodia may need to extend to reach areas to adhere in the vitreous, which is mostly water and a matrix of collagen fibers and several other compounds. Reduced filopodial number may also reduce the likelihood of contact with spaced collagen fibrils in the vitreous. We found evidence that reduction in filopodial number was associated with reduced intravitreous neovascularization and with reduced migration in the vertical migration assay and consistent with this, neither number of filopodia nor migration was reduced in the horizontal migration assay. However, the gradient effect of VEGF may not have been maintained in the horizontal migration assay and there was not a means to measure filopodia length or number in the vertical assay.

Filopodia extension toward an intraretinal gradient with reduced signaling through VEGFR2 may permit endothelial cells to migrate into the nearby retina by signaling through other receptors or integrins. Cell migration and other aspects of angiogenesis is reported to occur through signaling of VEGFR1, although we found no evidence of a change in VEGFR1 phosphorylation following injection of SU5416(Kami *et al*. 2008;Shih et al. 2003). Also, binding of endothelial integrins by various growth factors can modulate the activation of receptor tyrosine kinases and affect downstream signaling(Serini *et al*. 2008). Integrins can also form intracellular complexes with signaling molecules and cytoskeletal proteins in response to ligand binding. The β3 integrin, for example, has been implicated in human proliferative angiogenic diseases of the retina in vivo (Friedlander *et al*. 1996). Binding of the extracellular domain of the β3 integrin was found necessary for integrin phosphorylation and for VEGF induced phosphorylation of VEGFR2 (Mahabeleshwar *et al*. 2007), but inhibition of the receptor tyrosine kinase of VEGFR2 did not interfere with this event (Borges et al. 2000). The extracellular domain of β3 integrin also binds and phosphorylates platelet derived growth factor (PDGF)-Rβ and this binding enhances cell migration in vitro (Borges et al., 2000). It is possible through such a mechanism that inhibition of filopodial length may reduce intravitreous guidance cues for endothelial tip cells but not affect endothelial adhesion, activation of integrins or other receptors within the retina that would lead to VEGF gradient-directed cell migration toward the ora serrata. Although SU5416 inhibits the intracellular tyrosine kinase domain of VEGFR2 that is necessary for the internalization and ubiquitination of the receptor (Ewan *et al*. 2006), we did not find that free VEGF was reduced in the retina of SU5416 treated eyes compared to control. Our data showed that β3 integrin phosphorylation was not inhibited by a dose of SU5416 that caused inhibition of VEGFR2 phosphorylation and IVNV.

We did not find an increase in apoptotic cells from using the VEGFR2 inhibitor compared to control. However, more data are needed to determine safety, because inhibiting VEGF can have adverse effects on neuronal survival (Laudenbach *et al*. 2007;Oosthuyse, et al., 2001;Nishijima, et al, 2007) and SU5416 was shown to reduce the thickness of the retina and ganglion cell layer in the developing mouse retina (Robinson *et al*. 2001).

In summary, our study provides support that inhibition of the receptor tyrosine kinase of VEGFR2 using the small protein inhibitor, SU5416, can reduce IVNV and filopodial length in endothelial tip cells. We also provide strong evidence that intraretinal endothelial migration is not adversely affected. Our findings support that removing the guidance cues by inhibiting filopodial length and number should be further studied (Gupton S.L. and Gertler F.B. 2007) to provide insight into reducing IVNV without interfering with intraretinal vascularization, which is ongoing in the developing preterm infant.

ACKNOWLEDGEMENTS

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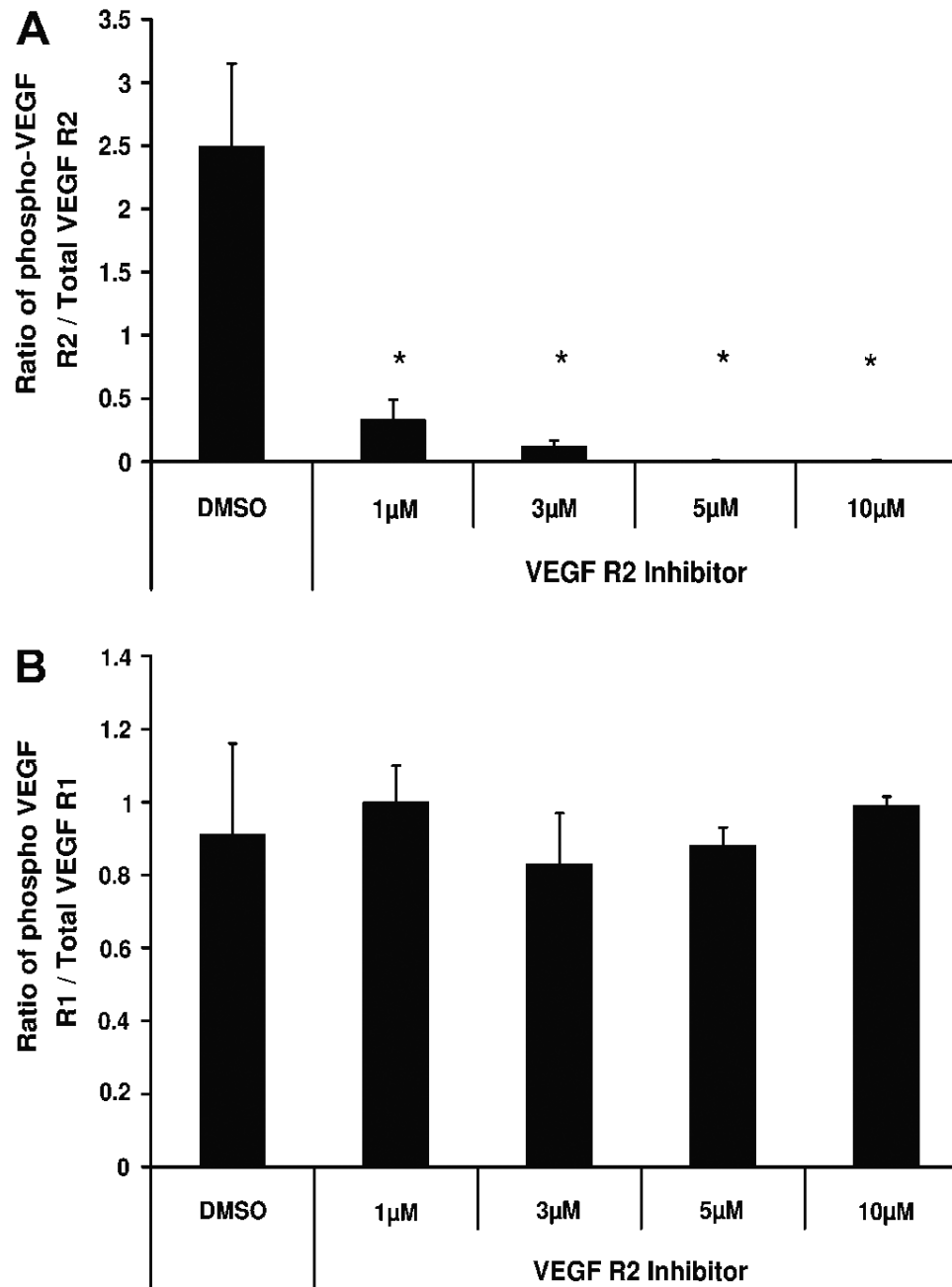
Figure 1.
Histogram showing phosphorylation of VEGF receptors in cultured HUVECs treated with different concentrations of VEGFR2 inhibitor (SU5416) or control, in the presence of VEGF. (A) Protein samples probed for phospho-VEGFR2 and reprobed for total VEGFR2 showed a dose dependent decrease in VEGFR2 phosphorylation (ANOVA, p=0.001, post-hoc testing for each dose of VEGFR2 inhibitor p≤ .005 compared to DMSO control). (B) Samples probed for phosho-VEGFR1 and reprobed with total VEGFR1 showed no response to SU5416. n=3/ condition.
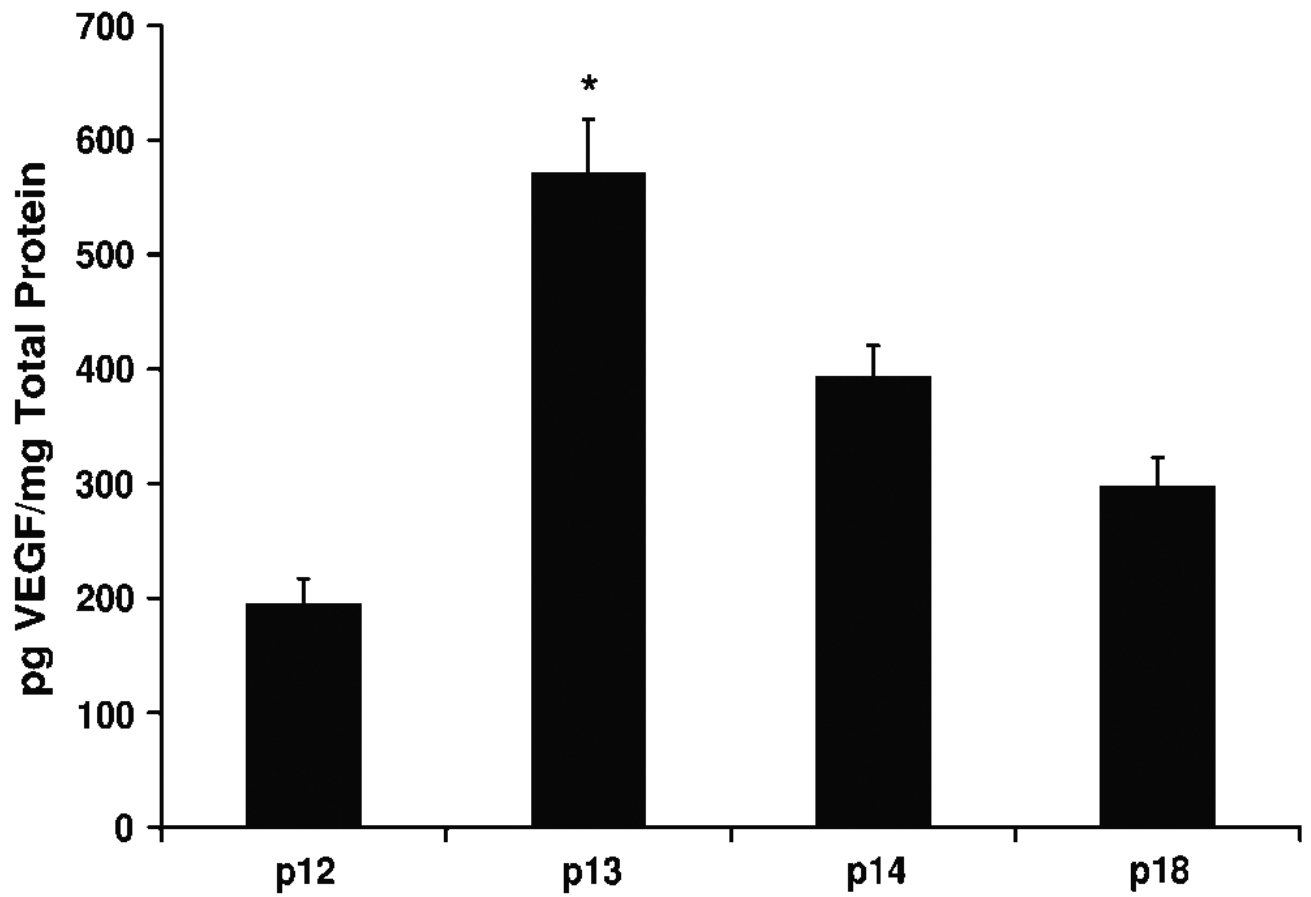
Figure 2.
VEGF ELISA of protein from retinas of p12, p13, p14, and p18 OIR rat pups. VEGF protein was significantly higher in p13 pups than all other time points (Anova $p<.001$, post hoc testing between p13 and all others, $^*p \leq .007$) n=5-7 for all timepoints.
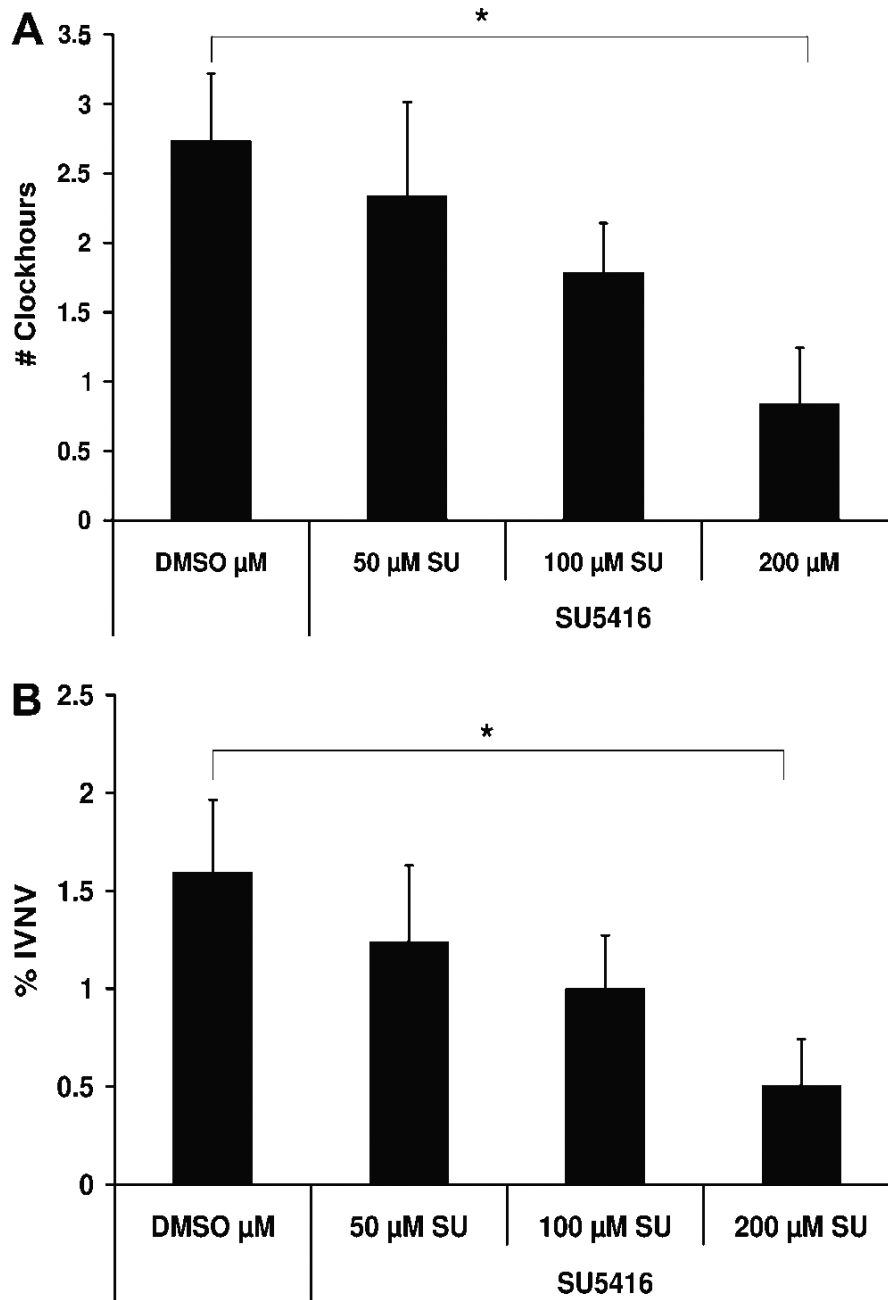
Figure 3.
Lectin stained retinal flat mounts from pups injected at p12 with SU5416 (50, 100, or 200 μM) or .5% DMSO were analyzed at p18. SU5416 had no effect on avascular area at any dose. (A) The inhibitor reduced the number of clockhours of intravitreous neovascularization (IVNV) at the highest concentration (200 μM) (Student T-test, p=.02 DMSO vs. 200 μM VEGFR2 Inhibitor). (B) SU5416 (200 μM dose) reduced IVNV compared to DMSO control at p18 in the rat 50/10 OIR model (Student T-test, p=.02 DMSO vs. 200 μM SU5416). n=10/ condition from at least 2 different litters.
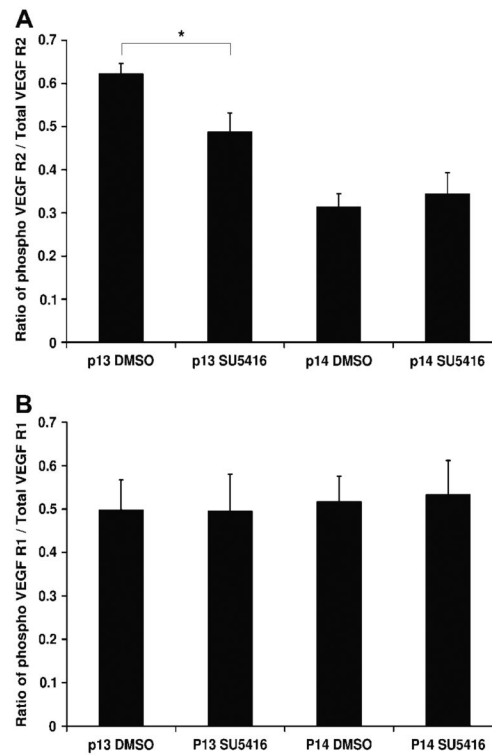
Figure 4.
Retinas from eyes injected at p12 with 200 μM SU5416 or DMSO control were taken at p13 and p14, homogenized, immunoprecipated for VEGFR1 or VEGFR2, and immunoblotted for phospho VEGFR1 and VEGFR2, respectively. (A) The VEGFR2 Inhibitor reduced levels of phosphorylated VEGFR2 in retinas from p13 pups, but not in p14 (Student T-test, *p=.019). (B) No change in phosphorylation of VEGFR1 was seen in p13 or p14 pups. n=6 for all treatments.
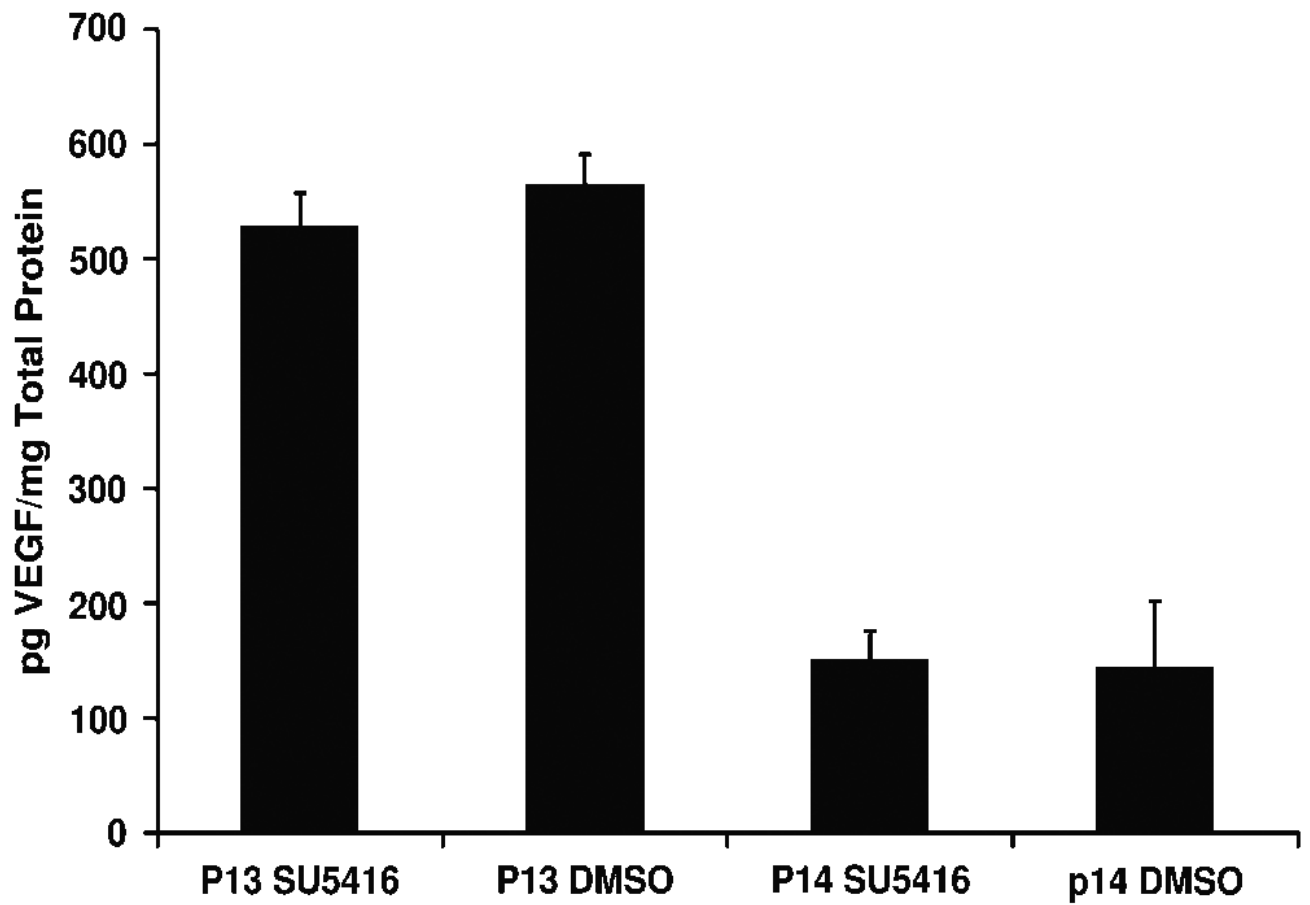
Figure 5.
VEGF ELISA of protein from retinas of p13 and p14 pups injected at p12 with 200 µM of SU5416 or .5% DMSO. No difference in VEGF was seen in retinas from pups injected with SU5416 or DMSO within a timepoint. (ANOVA, p>.05)
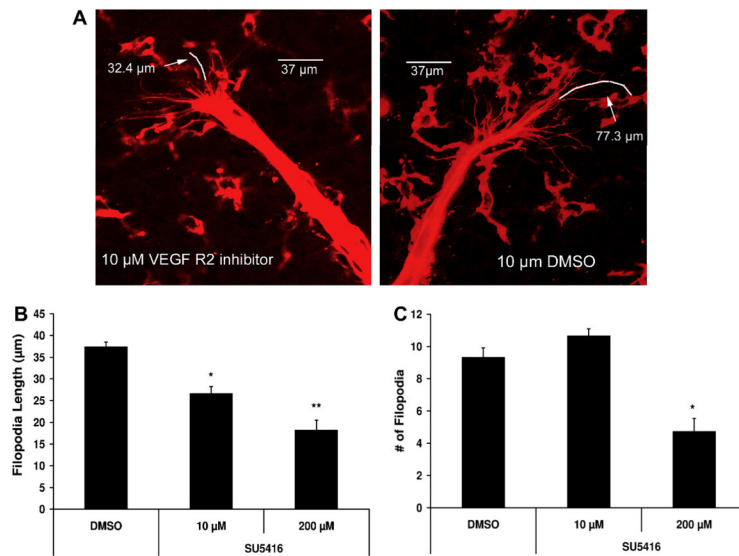
Figure 6.
Retinas at p14 from pups in 50/10 OIR model injected with SU5416. Lectin-stained flat mounts were analyzed for filopodia number and length/endothelial tip cells using ImageTool. (A) Image of tip cells along the leading edge of vascularization from retinas of eyes injected with SU5416 (10 μM and 200 μM) or DMSO. (B) Average lengths of filopodia were decreased by both SU5416 concentrations compared to DMSO control (ANOVA, $p < 0.001$, post hoc testing between 10 μM SU5416 and DMSO, *$p=0.001$, between 10 μM and 200 μM VEGF R2, **$p=0.005$). (C) No effect was seen in the number of filopodia/tip cell with 10 μM SU5416, but numbers were reduced with 200 μM (ANOVA $p<.001$, post hoc testing between 200 μM and DMSO control, *$p=0.001$). n=5 retinas, 16 tips cells per retina were averaged.
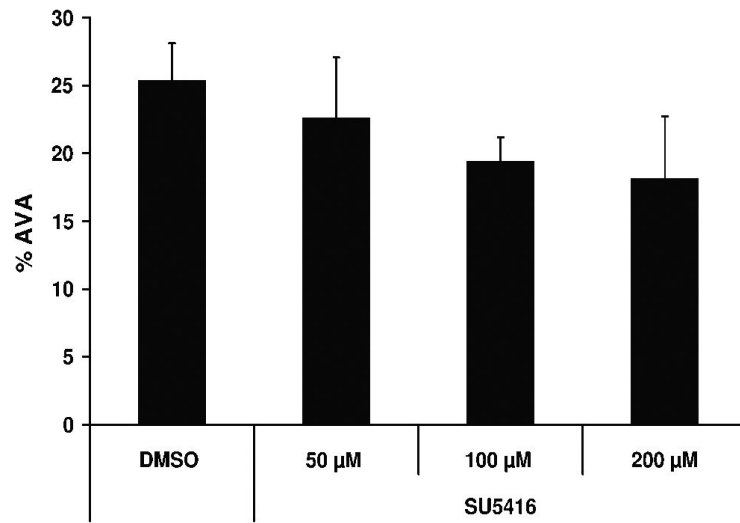
Figure. 7.
Lectin stained retinal flat mounts from pups injected at p12 with SU5416 (50, 100, or 200 μM) or .5% DMSO were analyzed at p18. SU5416 had no effect on avascular area at any dose (Student T-test, p=.19)
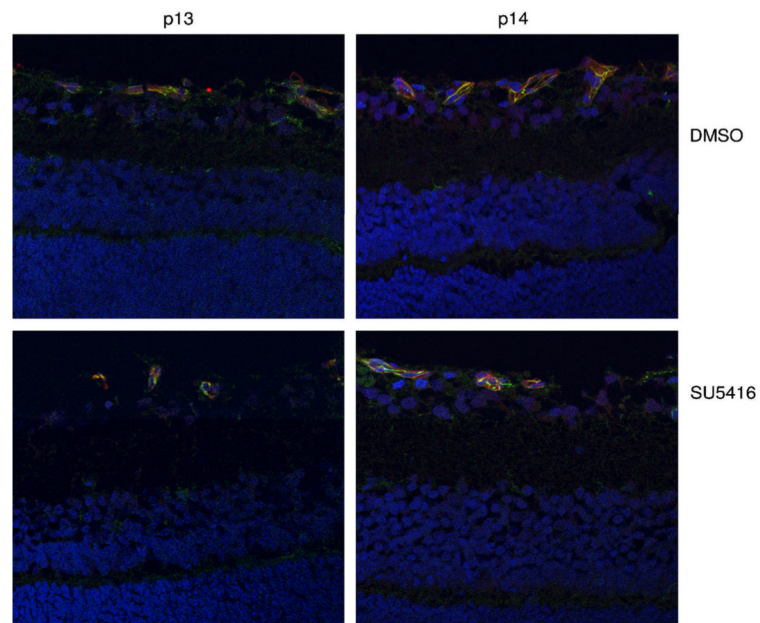
Figure 8.
Phosphorylation of β3-integrin appeared unchanged with retinas treated with SU5416. VEGFR2 injected, DMSO injected, and non-injected all moderately expressed phospho-β3-integrin (2+) at p13 and p14. Green fluorescence indicates phospho β-3 integrin, red indicates lectin, and blue is a Hoechst nuclear stain.
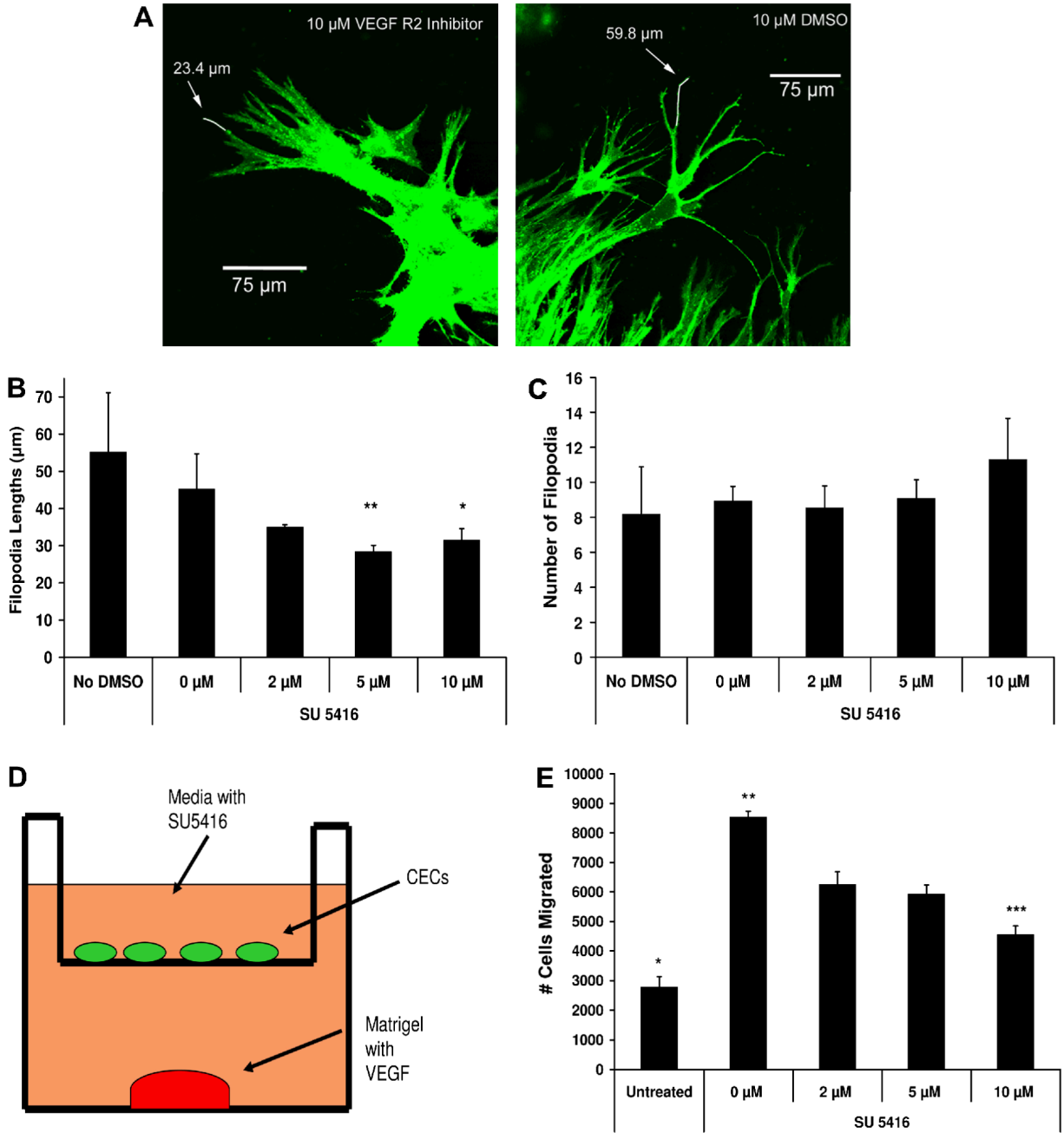
Figure 9.
Migration of human choroidal endothelial cells (ECs) was measured using two different assays. ECs were prestained with Vybrant DiO before being plated. ECs were treated with 0, 2, 5, or 10 µM of SU5416 in DMSO.

For Horizontal directed EC migration toward a VEGF gradient, (A) through (C), all groups were treated with 50 ng/mL VEGF: (A) Representative ECs with measured filopodia. (B) Filopodia length was reduced in ECs treated with SU5416. (ANOVA, p=0.03, post-hoc testing between 0 and 5 µM SU5416, ** p=0.01; between 0 and 10 µM SU5416, * p=0.02). Only filopodia that measured 10 µm long and less than 0.75 µm wide were used for analysis. (C) No

effect was seen on the number of filopodia per cell (ANOVA, p=0.16). n=10 cells/well averaged from 3 wells for each condition.

For Modified Boyden cell migration assay (Vertical directed EC migration toward a VEGF gradient), all groups were exposed to the same concentration of DMSO in media, including the untreated control. (D) Overview of cell migration assay: A matrigel spot was plated in each well with VEGF. A matrigel layer with no VEGF served as a negative control (untreated). (E) EC migration was significantly lower in cells without VEGF (untreated) compared to all treatments. The 0, 2, 5, and 10 µM doses of SU5416 all contained 50 ng/mL of VEGF. Migration was also inhibited by all concentrations of SU5416 (ANOVA, p<. 001, post hoc between control and treatments, *p<.015, between DMSO and treatments, **p≤ .001, between 10 µM and 2 µM, ***p=.021). n=5/treatment, repeated 3 times.