CD34 Promotes Pathological Epi-Retinal Neovascularization in a Mouse Model of Oxygen-Induced Retinopathy

Martin J. Siemerink¹,2,3, Michael R. Hughes⁴,⁵, Marchien G. Dallinga¹,2,3, Tomek Gora¹,2,3, Jessica Cait⁵,⁶, Ilse M. C. Vogels¹,2,3, Bahar Yetin-Arik¹,2,3, Cornelis J. F. Van Noorden¹,2,3, Ingeborg Klaassen¹,2,3, Kelly M. McNagny⁴,⁵,⁶, Reinier O. Schlingemann¹,2,3 *

¹ Ocular Angiogenesis Group, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands, ² Department of Ophthalmology, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands, ³ Department of Cell Biology and Histology, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands, ⁴ Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada, ⁵ The Biomedical Research Centre, University of British Columbia, Vancouver, BC, Canada, ⁶ Department of Experimental Medicine, University of British Columbia, Vancouver, BC, Canada

* r.schlingemann@amc.uva.nl

Abstract

The sialomucins CD34 and podocalyxin (PODXL) are anti-adhesive molecules expressed at the luminal membrane of endothelial cells of small blood vessels and facilitate vascular lumen formation in the developing mouse aorta. CD34 transcript and protein levels are increased during human angiogenesis, its expression is particularly enriched on endothelial tip cell filopodia and CD34 is a marker for tip cells in vitro. Here, we investigated whether CD34 merely marks endothelial tip cells or has a functional role in angiogenesis. We assessed that silencing CD34 in human microvascular endothelial cells has little effect on endothelial cell migration or invasion, but has a significant effect on vascular-endothelial growth factor-induced angiogenic sprouting activity in vitro. In vivo, the absence of CD34 reduced the density of filopodia on retinal endothelial tip cells in neonatal mice, but did not influence the overall architecture of the retinal vascular network. In oxygen-induced retinopathy, Cd34−/− mice showed normal intra-retinal regenerative angiogenesis but the number of pathological epi-retinal neovascular tufts were reduced. We conclude that CD34 is not essential for developmental vascularization in the retina, but its expression promotes the formation of pathological, invasive vessels during neovascularization.

Introduction

Angiogenesis is the process by which vessels grow and branch from existing vessels to generate new vascular beds (reviewed in ref [1]). Physiological angiogenesis in adults is critical for wound healing, reproduction, and tissue growth and regeneration. Accordingly, insufficient or pathological angiogenesis underlies many human diseases including myocardial infarction,
delayed wound healing (ulcerative disease), neurological disease, impairment of vision (proliferative retinopathies) and tumor growth and metastasis (reviewed in ref [2]). Thus, a more complete understanding of the mechanisms regulating both physiological and pathological angiogenesis may have clinical implications for the treatment of disease and advancement of regenerative therapies.

The highly organized vascular sprouts in angiogenesis are composed of distinctly differentiated endothelial cell subtypes that act in a strict hierarchical fashion [3, 4]. Among these subtypes are endothelial tip cells, the leading cells of vascular sprouts that form filopodia to aid migration towards a source of growth factors such as vascular endothelial growth factor-A (VEGF-A); and, to direct adjacent endothelial cells to form and elongate the stalk of sprouting vessels [4–8]. In addition, tip cells have other functions that are essential for capillary sprouting such as a specific proteolytic machinery that is required for migration and invasion into the extracellular matrix or ischemic areas [9, 10]. Whether tip cell functions differ in sprouting angiogenesis under normal conditions and pathological conditions is unknown.

CD34 and podocalyxin (PODXL) are transmembrane anti-adhesive sialomucins that are ubiquitously expressed on the luminal surface of endothelial cells in capillaries [11–15]. CD34 and PODXL also act as anti-adhesive molecules during lumen formation in the developing mouse aorta by maintaining or promoting the separation between contralateral apical endothelial cell lumen surfaces [16]. It has been shown in Cd34 knockout (Cd34−/−) mice that, when expressed on blood cells, CD34 enhances the adhesion, mobility and invasiveness of hematopoietic progenitors, mast cells, and eosinophils [17–20]. High expression of CD34, but not PODXL, on endothelial tip cells and their filopodia [4, 21] suggests that CD34 may play a role in angiogenesis that is specifically related to filopodia functions or architecture [7, 13, 22, 23]. A role of CD34 in tumor angiogenesis was suggested by the observation that Cd34 deletion in mice (specifically in non-hematopoietic lineages) impaired early tumor growth due to a delay in angiogenesis [24]. Whether this effect is specifically related to expression of CD34 on tip cells is not known. We have found that only a subset (approximately 10%) of cultured human umbilical vein endothelial cells (HUVECs) express CD34 and this CD34-positive population has a distinct endothelial tip cell phenotype [21]. We also showed that this subpopulation of CD34+ tip cells is actively restored when isolated CD34+ HUVECs are re-cultured [21]. Furthermore, stimulation of HUVECs with angiogenic growth factors such as VEGF-A induced CD34 expression [25]. Therefore, high CD34 expression marks endothelial cells with tip cell activity in vitro. However, it is not known whether CD34 has a functional role in tip cell behavior or angiogenesis in general.

In the following study, we investigated the role of CD34 in angiogenesis using in vitro angiogenesis models in the presence or absence of CD34-specific small interfering RNA (siRNA) and in physiological and pathological angiogenesis in vivo using Cd34−/− mice. In a model of oxygen-induced retinopathy (OIR), we provide evidence that, although CD34 is not necessary for physiological retinal blood vessel development, it promotes epi-retinal tuft formation in pathological retinal angiogenesis.

Materials and Methods

Cells and cell cultures

Immortalized human microvascular dermal endothelial (HMEC-1) cells were grown on culture flasks coated with 2% gelatin containing M199 medium (Gibco, Grand Island, NY, USA) supplemented with 5% human serum (Academisch Medisch Centrum Hospital, Amsterdam, The Netherlands), 5% fetal bovine serum (FBS) (Biowhittaker, Walkersville, MD, USA) and 1%
penicillin-streptomycin-glutamine (Gibco). HMEC-1 cells between passages 30 and 40 were used for all experiments. All cells were grown in a humidified 37°C incubator with 5% CO₂.

**Small interfering RNA (siRNA) transfection**

HMEC-1 were transfected with either CD34-specific siRNA pool or non-targeting siRNA (Accell SMARTpool, Dharmacon, Lafayette, CA, USA). The siRNAs were transfected using the reverse-transfection method, according to the manufacturer’s protocol. Cells were either collected at 72 h after transfection for RNA or spheroids, formed 52 h after transfection, were embedded in collagen gels (72 h after transfection) for sprouting assays.

**FACS and flow cytometry**

For flow cytometric analysis, HMEC-1 were labeled with anti-human podocalyxin antibody (goat pAb IgG, AF1556, R&D Systems, Minneapolis, MN, USA), anti-human CD34 antibody (mouse mAb IgG1, QBend10 clone, R&D Systems) or the appropriate isotype control antibody at the same concentrations followed by a fluorochrome-conjugated secondary antibody (Alexa Fluor® 647 chicken anti-goat IgG, A21469; ThermoFisher Scientific, Waltham, MA, USA) or fluorescein isothiocyanate (FITC) goat anti-mouse IgG (1010-02; Southern Biotech, Birmingham, AL, USA). Data was collected on an LSR II instrument (Becton Dickinson (BD), Mountain View, CA, USA) and analyzed using FlowJo v10.0.08 software (FlowJo, Ashland, OR, USA). For cell-sorting, HMEC-1 were labeled with anti-CD34 antibody (QBend10 clone; Sanquin, Amsterdam, The Netherlands) and sorted for CD34 expression on a FACSAria or FACS-Canto-II instrument (Becton Dickinson, Mountain View, CA, USA) as described previously [21].

**RNA isolation and gene expression analysis**

Total RNA was isolated from cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Approximately 1 μg of total RNA was used for DNase treatment (amplification grade; Invitrogen) and reverse transcription into first strand cDNA using Superscript III and oligo(dT)12–18 (Invitrogen). The primers used for the CD34 quantitative PCR (qPCR) analysis were 5’-GG AGCCAGGCTGATGGTGATG-3’ (For); 5’-ATCCCCAGCTTTTTCAGTCAGAT-3’ (Rev). NCBI BLAST confirmed specificity of the primers. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on agarose gels. Non-template controls were included to verify the method and the specificity of the primers. Mean primer efficiency was 96% ± 3%. Real-time qPCR was performed as described previously [26], using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).

**Spheroid-based angiogenesis model**

Endothelial cells (750 cells/spheroid) were seeded in medium containing methylcellulose (Sigma-Aldrich, St Louis, MO, USA) to form spheroids [27]. After 24 h, cells were embedded in collagen gel in the presence or absence of 50 ng/ml human recombinant VEGF-A (Sanquin) and allowed to sprout for 24 h. At least 8 spheroids per group were analyzed under an inverted microscope (Leica Microsystems, Mannheim, Germany) and phase contrast images were quantified using image analysis and ImageJ software [28].
Scratch assay

HMEC-1 were transfected with siRNAs as described above and cultured in 12-well tissue culture plates until confluent. The confluent monolayer was scraped with a 200-μl pipette tip to generate a wound and was rinsed twice with medium. Micrographs were taken at 40× magnification using an inverted microscope (Leica Microsystems) and phase contrast images were quantified using image analysis and ImageJ software [28].

Cell invasion assay

HMEC-1 were transfected with siRNAs as described above and seeded (5×10⁴ cells per well) on transwell Boyden chamber inserts (8 μm pores; Corning, Lowell, MA, USA) containing a polycarbonate filter as previously described [29]. Filters were pre-coated with Matrigel (BD Discovery Labware, Bedford, MA, USA) diluted 1:3 in M199 basal medium to create an artificial internal limiting membrane. Inserts containing cells were placed in M199 supplemented with 2% human serum in 24-well plates. HMEC-1 migration was stimulated by adding complete medium to the lower well of the Boyden chamber. After 24 h, membranes were washed with ice-cold phosphate-buffered saline (PBS) (Lonza, Walkersville, MD, USA) and the upper surface of the insert was swabbed to remove non-migrated cells. Cells that had migrated through the pores of the filter were either fixed in 4% paraformaldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA, USA) and stained with Hoechst or fixed with absolute methanol and stained with Giemsa. Migration was evaluated as the mean number of migrated cells in 5 high-power fields (HPF) per well (20× magnification). Each condition was assayed in triplicate and each experiment was performed at least twice.

Mice

All mice used in this study were backcrossed to C57Bl/6J mice for more than 12 generations. Cd34⁻/⁻ mice were generated as described previously [30]. Mice were bred and maintained in specific pathogen-free conditions at the Biomedical Research Centre (The University of British Columbia (UBC), Vancouver, BC, Canada). All animal experiments were approved by UBC’s animal care committee and were conducted humanely following institutional and Canadian Council on Animal Care guidelines (Protocol #A11-0289). Mice were sacrificed humanely by carbon dioxide inhalation at a flow rate of 20–30% chamber volume/min as per UBC ACC SOP.

Oxygen-induced retinopathy model and analysis of postnatal retinal angiogenesis

The OIR model was carried out as previously described [31] with the use of a BioSpherix ProOX A chamber equipped with ProOx P110 oxygen controller (BioSpherix, Lacona, NY, USA). Postnatal day 7 (P7) pups together with their nursing dams were placed for 5 consecutive days in a 75% oxygen chamber. Litters of 8 pups or less had 1 nursing dam; those with more than 8 pups had 2 nursing dams. The chamber was only opened briefly between P7 and P12 when the nursing dams were replaced with foster dams to mitigate any adverse effects of hyperoxia on the nursing dams. At P12, the pups were returned to room air (21% oxygen). Pups were sacrificed and their eyes were collected at P12, P17 and P21. In addition, mouse eyes were collected from pups raised in room air at P1, P3, P5, P7, P9 and P25 as controls.

Eyes were fixed for 30 min in 4% paraformaldehyde prepared in PBS. Retinas were dissected, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated in methanol and stored in methanol at -20°C. Before immunofluorescence analysis, retinal whole-mounts were
rehydrated, permeabilized in PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich) at 4°C overnight and washed with PBS. Retinal whole-mounts were blocked in PBlec (PBS (pH 6.8) with 1% Triton X-100, 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM MnCl₂ (all from Sigma-Aldrich)), and incubated with AF488-labeled or AF594-labeled lectin from *Bandeiraea simplicifolia* (isolectin B4) (Invitrogen) in PBlec at 4°C overnight. After extensive washing in PBS, the retinas were either flat mounted in Vectashield (Vector, Burlingame, CA, USA) or processed for multiple labeling, using primary antibodies directed against mouse CD34 (clone RAM34; eBioscience, San Diego, CA, USA) or mouse PODXL (clone 192704, R&D Systems). Secondary antibodies used were cyanine-3 (Cy3)-labeled donkey anti-goat and Cy3-labeled goat anti-rat, respectively (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Images were taken using a wide-field fluorescence microscope or confocal microscope (Leica Microsystems). In control eyes, filopodia at the vascular front were analyzed in 20 microscopic fields selected randomly from 5 retinas per group of mice (mutants or wild type littermates) and quantified using image analysis and ImageJ software [28]. For the OIR model, the retinal avascular areas and neovascularization areas were quantified using Adobe Photoshop CS4 software according to a published protocol [28, 32].

**Statistical analysis**

Values are given as mean values ± SD or SEM, as indicated. Data are represented as averages of independent experiments, performed in duplicate or triplicate. Statistical analyses were performed using the Student’s t-test or two-way ANOVA and P-values < 0.05 were considered to indicate statistically significant differences.

**Results**

**CD34 marks VEGF-responsive endothelial tip cells**

We previously reported that CD34 marks cells with an endothelial “tip cell” phenotype and gene expression pattern in cultures of HUVEC [21]. To evaluate a possible functional role of CD34 in endothelial cells during angiogenesis, we carried out *in vitro* experiments using HMEC-1. Similar to HUVEC [21], approximately 9–10% of HMEC-1 express high levels of CD34 (Fig 1A, right panel). To test the functional contribution of CD34⁺ and CD34⁻ cells to sprouting, we generated spheroids of FACS-isolated populations of CD34⁺ or CD34⁻ HMEC-1 and embedded them in collagen gels in the presence or absence of VEGF-A. After 24 h, CD34⁺ sorted HMEC-1 showed a significant increase in the number of sprouts (but not sprout length) in response to VEGF-A, as did spheroids composed of unsorted HMEC-1 cells. The CD34⁻ HMEC-1 spheroids were unresponsive to VEGF-A (Fig 1A and 1B). This shows that VEGF-A-responsive angiogenic sprouting activity in HMEC-1 cultures is associated with the CD34⁺ population.

**CD34 promotes VEGF-induced angiogenic sprouting *in vitro***

Although VEGF-A induced sprouting activity is associated with the CD34-expressing HMEC-1 population, it is possible that CD34 is simply a marker of “tip” like cells but does not serve a functional role. Therefore, to further investigate a possible functional role of CD34 in HMEC-1 sprouting angiogenesis or migration activity *in vitro*, we silenced CD34 expression in HMEC-1 using siRNA. Although only 10% of HMEC-1 in culture is positive for CD34, all cells express the closely related sialomucin podocalyxin (PODXL) (Fig 2A). Both CD34 and PODXL are widely expressed at the luminal plasma membrane of vascular endothelial cells. Knockdown of
Fig 1. The CD34+ fraction of HMEC-1 cultures contains the VEGF-induced angiogenic sprouting activity. (A, left panel) Representative images of spheroids that were generated from HMEC-1 were either unsorted or FACS-sorted, based on CD34 cell surface expression (CD34− and CD34+ populations). Spheroids were embedded in collagen gel supplemented with (+) or without (-) VEGF. Scale bar = 200 μm. (A, right panel) Flow cytometry dot plot demonstrating gating for HMEC-1 sorting based on CD34 expression. (B) The number of sprouts per spheroid and the mean sprout length were quantified using Image J. Error bars represent standard deviation. *Significantly different from unstimulated control (VEGF−) with P < 0.05.

doi:10.1371/journal.pone.0157902.g001
Fig 2. Silencing of CD34 expression in HMEC-1. (A) Representative histograms showing the intensity of anti-CD34 (left panel) or anti-podocalyxin (right panel) labeling of parental HMEC-1 compared to an isotype control (grey peak). (B) Quantitative PCR (qPCR) analysis of CD34 and PODXL mRNA levels and (C) flow cytometric analysis of CD34 protein expression on the membrane of HMEC-1 72 h after transfection with CD34-targeting (siCD34) or non-targeting (NT) (siNT) siRNA. Stimulation with VEGF induced CD34 plasma membrane expression in both siNT-treated and siCD34 treated HMEC-1. However, CD34 expression in siCD34-treated HMEC-1 was less than 30% as compared to expression levels in siNT-treated cells.

*Significantly different from non-stimulated cells with P < 0.05.

doi:10.1371/journal.pone.0157902.g002
CD34 by siCD34 was confirmed at the mRNA transcript level by qPCR (Fig 2B, left panel). Notably, knockdown of CD34 did not alter gene expression of PODXL (Fig 2B, right panel). Flow cytometric analysis confirmed suppression of CD34 protein expression on the plasma membrane in the presence or absence of exogenous VEGF-A (Fig 2C).

To explore whether CD34 gene ablation affects endothelial cell sprouting, migration and invasion, we performed three separate in vitro assays. First, we repeated the spheroid-based sprouting assay as described in Fig 1 using siCD34 transfected HMEC-1. Silencing of CD34 expression increased the number of sprouts per spheroid marginally (1.3-fold) in the absence of exogenous VEGF-A. However, spheroids in which CD34 was silenced did not respond to VEGF-A stimulation (Fig 3A). No significant differences were observed for sprout length when comparing spheroids silenced for CD34 with control spheroids (Fig 3A) and the results are in line with spheroids of FACS-isolated populations of CD34+ or CD34− HMEC-1. Second, we performed a wound closure (scratch) assay using siCD34 transfected HMEC-1. This experiment showed that silencing of CD34 did not alter HMEC-1 migration (Fig 3B). Third, we seeded siCD34 transfected HMEC-1 into Boyden chambers containing membranes pre-coated with Matrigel to mimic the extracellular matrix of endothelium. Silencing of CD34 did not affect the level of HMEC-1 invasion after 20 hours in this assay (Fig 3C). Thus, although silencing of CD34 results in a marginal increase in spheroids sprouting, the most important result seems to be that HMEC-1 without CD34 expression do not respond to VEGF-A, although a basic level of sprouting is still intact. Migration and invasion of HMEC-1 were not affected by silencing of CD34.

CD34 is expressed on endothelial tip cells and tip cell filopodia in vivo

Since our in vitro assays suggested that CD34 has a role in regulating VEGF-induced sprouting activity, we next wished to determine whether CD34 has a role in sprouting angiogenesis in vivo. In retinal angiogenesis, blood vessels are formed in an organized and directional manner and offer a controlled and physiological model to study angiogenesis in vivo. Our first objective was to determine the expression pattern of CD34 in the mouse retina during postnatal vessel development.

Immunohistochemistry of whole-mount retinas harvested from P5 mouse pups showed that CD34 and PODXL are expressed on the vasculature in the developing retina. Although CD34 expression is limited to a select population in HUVEC and HMEC-1 cultures, CD34 is more widely expressed throughout microvasculature in humans and is expressed on filopodial of angiogenic tip cells during active angiogenesis [21]. Likewise, we found that in the developing retinal vessels of neonatal mice, CD34 is expressed on the filopodial extensions of tip cells at the angiogenic vessel front in addition to the expression throughout the advancing sprouts, phalanx and the lumen of the vessel stalk (Fig 4A). The distribution pattern of CD34 on endothelial cells in angiogenic tissues was similar to that of isolectin B4 (an endothelial marker used to visualize endothelial tip cells and their filopodia) (Fig 4A) [6, 7, 33–36]. Notably, PODXL is expressed only within the stalk (and phalanx) region of vessels and is absent from the filopodia (Fig 4B). These findings suggest that CD34 and PODXL have non-redundant functions in vascular endothelia.

CD34 deletion reduces the number of tip cell filopodia without perturbing retinal vasculature development

Since CD34 is an anti-adhesive molecule, we hypothesized that CD34 reduces adhesion of filopodia to the extracellular matrix or membranes of other cells, thereby facilitating extension of tip cell filopodia. To test this hypothesis, we examined the morphologic features of tip cells...
during retinal development under normoxic conditions in wild type and Cd34−/− mice. Detailed analysis of the vascular front revealed that filopodia density was reduced by 30% in Cd34−/− mice at P5 as compared to wild type mice (Fig 5).

To determine whether the reduction density of filopodia in Cd34−/− mice has consequences for the physiological development of retinal vessels, we studied the retinal vasculature in normoxic wild type and Cd34−/− mice at P25 (Fig 6A), when the superficial, intermediate and outer vascular plexus vessels are fully mature. Isolectin B4 staining of retinas of Cd34−/− mice revealed no abnormalities in development in any of the retinal vascular plexus layers.

Fig 3. Effect of CD34 silencing on sprouting, cell migration and invasion. (A, left panel) Representative images of spheroids that were generated from HMEC-1 transfected with either a non-targeting siRNA (siNT) or siCD34 and subsequently embedded in collagen gel in the presence or absence of VEGF-A. (A, right panel) Spheroids were analyzed at 24 h after embedding and the number of sprouts per spheroid and average sprout length were quantified using Image J. Results were expressed relative to values of siNT transfected cells without VEGF. (B, left panel) siNT- and siCD34-transfected HMEC-1 were grown until confluent. Scratches were made using a pipette tip and images were taken at 0 and 6 h after scratching. (B, right panel) The percentage of width of the scratch filled with cells was quantified over time. (C, left panel) Representative images of HMEC-1 transfected with siNT or siCD34, located at the lower side of the Boyden filter after invasion through Matrigel visualized by DNA staining (i,iii) or Giemsa (ii,iv) staining at 20 h after seeding. (C, right panel) Cells invading the Matrigel were quantified by counting the number of nuclei per microscopic field. Error bars represent standard deviation, except for Fig 3C (right panel) where they represent 95% confidence interval; *Significantly different from siNT control with P < 0.05.

doi:10.1371/journal.pone.0157902.g003
Although we did not detect PODXL expression on tip cell filopodia of advancing retinal vessels in wild type mice, it is possible that, in the absence of CD34, PODXL is expressed as a compensatory mechanism. However, immunohistochemistry of whole-mount retinas revealed no difference in PODXL expression on the vasculature in the developing retinas of wild type and Cd34−/− mice. Moreover PODXL staining was not detected in filopodia at the angiogenic front in Cd34−/− mice (Fig 6B).

From this data we concluded that, although the CD34 enhances formation of filopodia at the angiogenic front of retinal vessels, CD34 is dispensable for vascularization of the mouse retina. In addition, PODXL does not compensate for loss of CD34 expression during retinal angiogenesis. These data suggest that CD34 expression marks endothelial tip cells during retinal angiogenesis, but it is not functionally required for vessel development in the retina.

CD34 is involved in epi-retinal neovascularization but is dispensable for restoration of retinal vasculature following vaso-obliteration

We next used the OIR model to determine whether ablation of Cd34 impairs pathological retinal neovascularization. The OIR model is an acute bi-phasic model of pre-retinal neovascularization associated with ischemia in the retina that develops after a period of experimental hyperoxia. In the first phase, P7 mouse pups are exposed to a high oxygen concentration (75% O2) for 5 days (P7-P12) to cause retinal vaso-obliteration in the central posterior retina [37]. In the second phase, pups are returned to normoxia where the relatively low oxygen concentration of 21% causes hypoxia and consequent excessive angiogenesis beyond the retina into the vitreous. Endothelial cell migration and invasion through the inner limiting membrane (the
boundary between the neuroretina and the vitreous cavity) is an early phase of the formation of pathological neovascular tufts that expand beyond the retina in the OIR model.

We first analyzed the degree of vaso-obliteration in $Cd34^{-/-}$ and wild type pups after 5 days of hyperoxia (P12) and found that exposure to hyperoxia resulted in a significantly reduced number of retinal capillaries especially in the central area (Fig 7A) but the extent of vaso-obliteration was identical in wild type and $Cd34^{-/-}$ mice at P12 and P17 (Fig 7A and 7B). However, the ratio of the area of pre-retinal neovascularization to the total retinal area was significantly lower at P17 in $Cd34^{-/-}$ mice (7.8%) as compared to wild type mice (14.0%) (Fig 7C). Furthermore, confocal images of P17 OIR retinas labeled with isoelectin B4 revealed that epi-retinal tufts in wild type mice aggregated as large continuous areas of neovascularization whereas in $Cd34^{-/-}$ mice, the considerably smaller tufts did not aggregate (Fig 7D). At P25, vaso-obliteration or neovascularization was not detected in OIR-treated wild type or $Cd34^{-/-}$ mice and both genotypes appeared to have recovered the retinal vasculature at this stage (data not shown). Thus, in a model of pathological angiogenesis, the absence of CD34 reduces epi-retinal vessel tuft formation, a process that contributes to vision impairment in human retinopathies.
Earlier studies showed that CD34 is actively regulated on vascular endothelial cells and that angiogenic factors play a role in this regulation [7, 12, 13, 16, 21–23, 27, 38]. The role of VEGF in CD34 expression on endothelial cells *in vitro* has been controversial with studies showing that it either downregulates [12, 38] or upregulates [21, 27] expression. We hypothesized that the anti-adhesive function of CD34 is relevant in angiogenesis during invasion of endothelial tip cells through the basal lamina and other extracellular matrix structures. This hypothesis was derived from our previous discoveries that CD34 enhances adhesion, mobility and invasiveness of hematopoietic progenitors, mast cells, and eosinophils [17–20]. The observation of high expression of CD34 on endothelial tip cells and their filopodia suggests a similar molecular function [7, 13, 21–23]. Adhesive and anti-adhesive interactions between cell surface proteins and individual components of the vascular basal lamina determine the number and the
behavior of endothelial tip cells during angiogenesis [4]. We showed that sprouting angiogenesis activity in HMEC-1 cultures is enhanced in the CD34+ fraction of HMEC-1 cells, and silencing of CD34 expression in HMEC-1 cells inhibits their response to exogenous VEGF-A. Migration is a key characteristic of tip cells. However, we did not find any effect of silencing of CD34 on HMEC-1 invasion or migration in vitro. We conclude that the absence of CD34 prevents VEGF-induced angiogenic sprouting in vitro, but does not limit endothelial cell migration or invasion in vitro.

During developmental angiogenesis in the mouse retina, CD34 is expressed on filopodia of endothelial tip cells in a distribution pattern similar to that of isolectin B4 [22]. We show that ablation of CD34 in mice reduces the formation of filopodia in retinal tip cells by

---

Fig 7. Loss of CD34 limits formation of pathological neovascularization in oxygen-induced retinopathy (OIR). (A) Representative retinal images showing total retinal area and regions of vaso-obliteration (VO) and neovascularization (NV) at P17. Scale bar = 50 μm (B) Ratios of VO to total retinal area indicated no significant difference in VO between wild type and Cd34−/− mice. (C) At P17, neovascularization was significantly decreased in Cd34−/− mice as compared to wild type mice (*P < 0.01). Error bars represent standard deviation. * Significantly different from wild type with P < 0.05. (D) Isolectin B4 labeling of retinas harvested at P25 after OIR revealed that epi-retinal tufts in wild type mice aggregated as large continuous areas of neovascularization whereas in Cd34−/− mice, the considerably smaller tufts did not aggregate. Scale bar = 50 μm.

doi:10.1371/journal.pone.0157902.g007
approximately 30% during developmental retinal angiogenesis. However, this reduction in filopodia was not sufficient to cause a loss of vessel density in the fully developed retinas in Cd34−/− mice. This is consistent with observations by Sawamiphak et al [39] who report that reduction of filopodial density by at least 50% is needed to attenuate vessel density in the retina and Phng et al who show that filopodia are dispensable for angiogenesis [8]. Because we have previously shown that PODXL is a potent inducer of microvillus formation in epithelial cells [40] and because microvilli and filopodia share many structural similarities and molecular components, we considered the possibility that PODXL compensates for the loss of CD34 in the formation of endothelial tip cell filopodia. However, we did not detect PODXL expression on filopodia at the retinal vessel front in either wild type or Cd34−/− mice.

We found that CD34 did not impair physiological retinal angiogenesis aimed to restore the vasculature due to OIR. However, CD34 ablation impaired pathological epi-retinal tuft formation in the OIR model. Intriguingly, Budd et al. showed that reduced numbers of endothelial tip cell filopodia corresponded to a reduced intravitreal but not intra-retinal vascularization [41]. Therefore, it is possible that the diminished number of filopodia in retinal tip cells in Cd34−/− mice hampered intravitreous invasion. This suggests that CD34 has a functional role in VEGF-mediated vessel growth.

Conclusion

In conclusion, our study shows that CD34 is important for the formation of vascular sprouts and filopodia but CD34 does not appear to be essential for the development of an intact retinal vascular network. However, the absence of CD34 limits invasion of vessels into the vitreous and formation of epi-retinal tufts in the OIR model, which suggests that CD34 is involved in the pathological neovascularization causing vision loss in proliferative retinopathies.

Acknowledgments

We thank Drs. W. Song and F. Cai (Department of Psychiatry, Faculty of Medicine, University of British Columbia) for use of their mouse oxygen incubator. Thank you to the core staff and facilities of the Biomedical Research Centre for their support in providing media, animal care and genotyping services. We are grateful to Dr. Jennifer Baker for her helpful suggestions and critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: MJS MRH MGD CJFVN IK KMM ROS. Performed the experiments: MJS MRH TG MGD IMCV JC BY-A. Analyzed the data: MJS MRH CJFVN IK KMM ROS BY-A. Contributed reagents/materials/analysis tools: KMM ROS. Wrote the paper: MJS MRH CJFVN IK KMM ROS. Conceived and initiated the study: MJS KMM ROS.

References

1. Chung AS, Ferrara N. Developmental and pathological angiogenesis. Annual review of cell and developmental biology. 2011; 27:563–84. doi: 10.1146/annurev-cellbio-092910-154002 PMID: 21756109.
2. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011; 473(7347):298–307. doi: 10.1038/nature10144 PMID: 21593862; PubMed Central PMCID: PMC4049445.
3. Herbert SP, Stainier DY. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. NatRevMolCell Biol. 2011; 12(9):551–64.
4. Siemerink MJ, Klaassen I, Van Noorden CJ, Schlingemann RO. Endothelial tip cells in ocular angiogenesis: potential target for anti-angiogenesis therapy. JHistochemCytochem. 2013; 61(2):101–15.
5. Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol. 2004; 242(1):91–101.

6. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 2003; 161(6):1163–77.

7. Schlingemann RO, Rietveld FJ, de Waal RM, Bradley NJ, Skene AI, Davies AJ, et al. Leukocyte antigen CD34 is expressed by a subset of cultured endothelial cells and on endothelial abluminal microprocesses in the tumor stroma. Lab Invest. 1990; 62(6):690–6. PMID: 1694254

8. Phng L-K, Stanchi F, Gerhardt H. Filopodia are dispensable for endothelial tip cell guidance. Development. 2013; 140(19):4031–40. doi: 10.1242/dev.097352 PMID: 24046319

9. Arroyo AG, Iruela-Arispe ML. Extracellular matrix, inflammation, and the angiogenic response. Cardiovasc Res. 2010; 86(2):226–35. doi: 10.1093/cvr/cvq049 PMID: 20154066

10. Koziol A, Gonzalez P, Mota A, Pollan A, Lorenzo C, Colome N, et al. The protease MT1-MMP drives a combinatorial proteolytic program in activated endothelial cells. FASEB J. 2012; 26(11):4481–94. doi: 10.1096/fj.12-205906 PMID: 22659368

11. Baumhueter S, Dybdal N, Kyle C, Lasky LA. Global vascular expression of murine CD34, a sialomucin-like endothelial ligand for L-selectin. Blood. 1994; 84(8):2554–65. PMID: 7522633

12. Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, et al. Expression of the CD34 gene in vascular endothelial cells. Blood. 1990; 75(12):2417–26. PMID: 1693532

13. Ito A, Nomura S, Hirota S, Suda J, Suda T, Kitamura Y. Enhanced expression of CD34 messenger-RNA by developing endothelial cells of mice. Lab Invest. 1995; 72(5):532–8. PMID: 7538181

14. Nielsen JS, McNagny KM. CD34 is a key regulator of hematopoietic stem cell trafficking to bone marrow and mast cell progenitor trafficking in the periphery. Microcirculation. 2009; 16(6):487–96. doi: 10.1080/10739680902941737 PMID: 19479621

15. Testa JE, Chrastina A, Oh P, Li Y, Witkiewicz H, Czarny M, et al. Immunotargeting and cloning of two CD34 variants exhibiting restricted expression in adult rat endothelia in vivo. Am J Physiol Lung Cell Mol Physiol. 2009; 297(2):L251–L62. doi: 10.1152/ajplung.90565.2008 PMID: 19465515

16. Strilic B, Kucera T, Eglinger J, Hughes MR, McNagny KM, Tsukita S, et al. The molecular basis of vascular lumen formation in the developing mouse aorta. Developmental Cell. 2009; 17(4):505–15. doi: 10.1016/j.devcel.2009.08.011 PMID: 19853964

17. Blanchet MR, Maltby S, Haddon DJ, Merkens H, Zbytnuik L, McNagny KM. CD34 facilitates the development of allergic asthma. Blood. 2007; 110(6):2005–12. PMID: 17557898

18. Blanchet MR, Gold M, Maltby S, Bennett J, Petri B, Kubers P, et al. Loss of CD34 leads to exacerbated autoimmune arthritis through increased vascular permeability. J Immunol. 2010; 184(3):1293–9. doi: 10.4049/jimmunol.0900808 PMID: 20038636

19. Drew E, Merzaban JS, Seo W, Ziltener HJ, McNagny KM. CD34 and CD43 inhibit mast cell adhesion and are required for optimal mast cell reconstitution. Immunity. 2005; 22(1):43–57. PMID: 15664158

20. Maltby S, Woelfarth C, Gold M, Zbytnuik L, Hughes MR, McNagny KM. CD34 is required for infiltration of eosinophils into the colon and pathology associated with DSS-induced ulcerative colitis. Am J Pathol. 2010; 177(3):1244–54.

21. Siemerink MJ, Klaassen I, Vogels IM, Griffioen AW, Van Noorden CJF, Schlingemann RO. CD34 marks angiogenic tip cells in human vascular endothelial cell cultures. Angiogenesis. 2012; 15(1):151–63. doi: 10.1007/s10456-011-9251-z PMID: 22249946

22. Wood HB, May G, Healy L, Enver T, MorrisKay GM. CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. Blood. 1997; 90(6):2300–10. PMID: 9457674

23. Young PE, Baumhueter S, Lasky LA. The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. Blood. 1995; 85(1):96–105. PMID: 7528578

24. Maltby S, Freeman S, Gold MJ, Baker JHE, Minchinton AI, Gold MR, et al. Opposing roles for CD34 in B16 melanoma tumor growth after early stage vasculature and late stage immune cell infiltration. Plos One. 2011; 6(4):e18160. doi: 10.1371/journal.pone.0018160 PMID: 21494591

25. Korff T, Kimmina S, Martiny-Baron G, Augustin HG. Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. FASEB J. 2001; 15(2):447–57. PMID: 11569600

26. Klaassen I, Hughes JM, Vogels IM, Schalkwijk CG, Van Noorden CJ, Schlingemann RO. Altered expression of genes related to blood-retina barrier disruption in streptozocin-induced diabetes. Experimental eye research. 2009; 89(1):4–15. doi: 10.1016/j.exer.2009.01.006 PMID: 19284967.
27. Korff T, Augustin HG. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. J Cell Biol. 1998; 143(5):1341–52. PMID: 9832561; PubMed Central PMCID: PMCPMC2133072.
28. Chieco P, Jonker A, De Boer BA, Ruijter JM, Van Noorden CJ. Image cytometry: protocols for 2D and 3D quantification in microscopic images. Progress in histochemistry and cytochemistry. 2013; 47(4):211–333. doi: 10.1016/j.proghi.2012.09.001 PMID: 23146330.
29. Shankar S, Chen Q, Srivastava RK. Inhibition of PI3K/AKT and MEK/ERK pathways act synergistically to enhance antiangiogenic effects of EGCG through activation of FOXO transcription factor. J Mol Signal. 2008; 3(7). doi: 10.1186/1750-2187-3-7 PMID: 18355401
30. Suzuki A, Andrew DP, Gonzalo JA, Fukumoto M, Spellberg J, Hashiyama M, et al. CD34-deficient mice have reduced eosinophil accumulation after allergen exposure and show a novel crossreactive 90-kD protein. Blood. 1996; 87(9):3550–62. PMID: 8611677
31. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D’Amato R, Sullivan R, et al. Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci. 1994; 35(1):101–11. PMID: 7507904
32. Connor KM, Krah NM, Dennison RJ, Aderman CM, Chen J, Guerin KI, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. Nature Protocols. 2009; 4(11):1565–73. doi: 10.1038/nprot.2009.187 PMID: 19816419
33. Hughes S, Yang HJ, Chan-Ling T. Vascularization of the human fetal retina: Roles of vasculogenesis and angiogenesis. Invest OphthalmolVisSci. 2000; 41(5):1217–28.
34. Klosowiskii BN, Zhukova T. [Effect of colchicine on remote phases of growing capillaries in the brain]. ArkhPatol. 1963; 35(3):38–44.
35. Marin-Padilla M. Early vascularization of the embryonic cerebral cortex: Golgi and electron microscopic studies. J Comp Neurol. 1985; 241(2):237–49.
36. Schoefl GI. Studies on inflammation. III. Growing capillaries: Their structure and permeability. Virchows ArchPatholAnatPhysiol KlinMed. 1963; 337:97–141.
37. Stahl A, Connor KM, Sapieha P, Chen J, Dennison RJ, Krah NM, et al. The mouse retina as an angiogenesis model. Invest OphthalmolVisSci. 2010; 51(6):2813–26.
38. Hellwig SM, Damen CA, van Adrichem NP, Blijham GH, Groenewegen G, Griffioen AW. Endothelial CD34 is suppressed in human malignancies: role of angiogenic factors. Cancer Lett. 1997; 120(2):203–11. PMID: 9461038.
39. Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, et al. Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature. 2010; 465(7297):487–91. doi: 10.1038/nature08995 PMID: 20445540
40. Nielsen JS, Graves ML, Chelliah S, Vogl AW, Roskelley CD, McNagny KM. The CD34-related molecule podocalyxin is a potent inducer of microvillus formation. PLoS One. 2007; 2(2):e237. doi: 10.1371/journal.pone.0000237 PMID: 17311105; PubMed Central PMCID: PMCPMC1796660.
41. Budd S, Byfield G, Martiniuk D, Geisen P, Hartnett ME. Reduction in endothelial tip cell filopodia corresponds to reduced intravitreous but not intraretinal vascularization in a model of ROP. Exp Eye Res. 2009; 89(5):718–27.