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Attenuation of retinal vascular development and neovascularization in PECAM-1-deficient mice

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Abstract

Platelet–endothelial cell adhesion molecule-1 (PECAM-1/CD31) is expressed on the surface of endothelial cells (EC) at high levels with important roles in angiogenesis and inflammation. However, the physiological role PECAM-1 plays during vascular development and angiogenesis remains largely unknown. Here we determined the role of PECAM-1 in the postnatal development of retinal vasculature and retinal neovascularization during oxygen-induced ischemic retinopathy (OIR) using PECAM-1-deficient (PECAM-1−/−) mice. A significant decrease in retinal vascular density was observed in PECAM-1−/− mice compared with PECAM-1+/+ mice. This was attributed to a decreased number of EC in the retinas of PECAM-1−/− mice. An increase in the rate of apoptosis was observed in retinal vessels of PECAM-1−/− mice, which was compensated, in part, by an increase in the rate of proliferation. However, the development and regression of hyaloid vasculature were not affected in the absence of PECAM-1. We did not observe a significant defect in astrocytes, the number of endothelial tip cell filopodias, and the rate of developing retinal vasculature progression in PECAM-1−/− mice. However, we observed aberrant organization of arterioles and venules, decreased secondary branching, and dilated vessels in retinal vasculature of PECAM-1−/− mice. In addition, retinal neovascularization was attenuated in PECAM-1−/− mice during OIR despite an expression of VEGF similar to that of PECAM-1+/+ mice. Mechanistically, these changes were associated with an increase in EphB4 and ephrin B2, and a decrease in eNOS, expression in retinal vasculature of PECAM-1−/− mice. These results suggest that PECAM-1 expression and its potential interactions with EphB4/ephrin B2 and eNOS are important for survival, migration, and functional organization of EC during retinal vascular development and angiogenesis.

Keywords: CD31; Angiogenesis; Apoptosis; Retinal vascularization; Retinopathy of prematurity; Hyaloid vasculature; Retinal endothelial cells

Introduction

PECAM-1 is a member of the immunoglobulin gene superfamily of cell adhesion molecules. It is highly expressed on the surface of EC and at lower levels on the surface of platelets and hematopoietic cells. PECAM-1 is important in endothelial cell–cell interactions during monolayer formation in culture (Albelda et al., 1990) and capillary morphogenesis in Matrigel (Sheibani et al., 1997). Antibodies to PECAM-1 block angiogenesis in mouse corneal pocket assays (DeLisser et al., 1997). Furthermore, the important role of PECAM-1 in angiogenesis has been recently demonstrated in PECAM-1-deficient (PECAM-1−/−) mice. These mice exhibited defects in their angiogenesis and inflammatory responses to foreign body challenges (Graesser et al., 2002; Solowiej et al., 2003). However, the physiological role PECAM-1 plays during vascular development and angiogenesis requires further investigation.

The developing mouse retinal vasculature provides a unique opportunity to study all aspects of vascular development postnatally and is readily amenable to biochemical evaluations. Mice are born without any retinal blood vessels, which develop immediately after birth. During the first week of life, a superficial layer of vessels is formed, which sprouts perpendicularly

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deep into the retina forming the deep retinal vascular plexuses by the third week of postnatal life. Retinal vasculature continues to undergo pruning and remodeling during the next 3 weeks, and a complete vasculature is established by 6 weeks of age (Dorrell et al., 2002; Fruttiger, 2002; Michaelson et al., 1954; Wang et al., 2003).

An inherent characteristic of the developing retinal vasculature is its sensitivity to changes in oxygen levels. This is responsible for ischemia-driven retinal neovascularization, which occurs in children that are born prematurely and are exposed to high levels of oxygen in order to help with their breathing and lung development. The exposure to high oxygen results in decreased levels of proangiogenic factors promoting the loss of existing retinal blood vessels and preventing the growth of additional blood vessels. Therefore, as a result of this the retina becomes ischemic upon exposure to normal air inducing the expression of proangiogenic factors, such as VEGF. This promotes growth of new blood vessels. Unfortunately, these vessels are abnormal in function and are leaky. They grow into the vitreous where they hemorrhage resulting in retinal detachment and loss of vision. Thus, understanding the molecular and cellular mechanisms that impact retinal neovascularization will aid in the development of new strategies to intervene and prevent loss of vision.

Oxygen-induced ischemic retinopathy (OIR) in the mouse is a highly reproducible model of angiogenesis *in vivo* and recapitulates the human condition described above (Smith et al., 1994). In this model, postnatal day 7 (P7) mice are exposed to 75% oxygen for 5 days and then brought to room air for 5 days, during which time maximum retinal neovascularization occurs. To gain further insight into the physiological role PECAM-1 plays during vascular development and neovascularization, we compared the normal postnatal development of retinal vasculature and retinal neovascularization during OIR in PECAM-1+/+ and PECAM-1−/− mice. Here we demonstrate that PECAM-1−/− mice exhibit decreased retinal vascular density. This was mainly attributed to the enhanced rate of apoptosis and the decreased number of EC observed in retinal vasculature of the PECAM-1−/− mice. The retinal blood vessels were also dilated and had fewer secondary branches in these mice, perhaps through deregulated expression and/or activity of EphB4/ephrin B2 and eNOS. However, the development of the ocular embryonic (hyaloid) vessels and their regression, an apoptosis-dependent process, was not affected in PECAM-1−/− mice. Furthermore, retinal neovascularization was impaired in PECAM-1−/− mice during OIR. This was associated with the failure of PECAM-1−/− mice to up-regulate eNOS expression. These studies demonstrate an important role for PECAM-1 during normal development and remodeling of retinal vasculature and its neovascularization during OIR.

Materials and methods

Tissue preparation

The targeting of the *PECAM-1* gene and the generation of mutant mice in a C57BL/6 background were previously described (Duncan et al., 1999). PECAM-1−/− mice and wild-type mice were maintained at the University of Wisconsin animal facility and studies were performed according to approved protocols. Mice were bred for different experimental time points. For oxygen-induced ischemic retinopathy, 7-day-old (P7) pups and their mother were placed in an airtight incubator and exposed to an atmosphere of 75±0.5% oxygen for 5 days. Incubator temperature was maintained at 23±2 °C, and oxygen was continuously monitored with a PROOX model 110 oxygen controller (Reming Bioinstruments Co., Redfield, NY). Mice were brought to room air for 5 days, and then pups were sacrificed for retinal whole-mount preparations and neovascularization analysis as described below.

Trypsin-digested retinal vessel preparations

Eyes were enucleated from P21 or P42 mice and fixed in 4% paraformaldehyde for at least 24 h. The eyes were bisected equatorially and the entire retina was removed under the dissecting microscope. Retinas were washed overnight in distilled water and incubated in 3% trypsin (Trypsin 1:250, Difco) prepared in 0.1 M Tris, 0.1 M maleic acid, pH7.8 containing 0.2 M NaF for approximately 1–1.5 h at 37 °C. Following completion of digestion, retinal vessels were flattened by four radial cuts and mounted on glass slides for periodic acid-schiff (PAS) and hematoxylin staining. Nuclear morphology was used to distinguish pericytes from EC. The nuclei of EC are oval or elongated and lie within the vessel wall along the axis of the capillary, while pericyte nuclei are small, spherical, stain densely and generally have a protuberant position on the capillary wall. The stained and intact retinal whole mounts were coded, and subsequent counting was performed masked.

The numbers of EC and pericytes were determined by counting respective nuclei under the microscope at a magnification of ×400. A mounting reticle (10 μm×10 μm) was placed in one of the viewing oculars to facilitate counting. Only retinal capillaries were included in the cell count, which was performed in the mid-zone of the retina. We counted the number of EC and pericytes in four reticles from the four quadrants of each retina. The total number of EC and pericytes for each retina was determined by adding the numbers from the four reticles. The ratio of EC to pericytes was then calculated. To evaluate the density of cells in the capillaries, the mean number of EC or pericytes was recorded in four reticles from each of the four quadrants of each retina.

Visualization of retinal vasculature and quantification of the rate of vascular expansion and avascular areas during OIR

The retinal vascular pattern and vessel obliteration were analyzed using retinal whole mounts stained with anti-collagen IV antibody as described previously (Wang et al., 2003). At various times mouse eyes were enucleated and briefly fixed in 4% paraformaldehyde (10 min on ice). The eyeballs were fixed in 70% ethanol for at least 24 h at −20 °C. Retinas were dissected in PBS and then washed with PBS three times, 10 min each. Following incubation in blocking buffer (50% fetal calf serum, 20% normal goat serum in PBS) for 2 h, the retinas were incubated with rabbit anti-mouse collagen IV (Chemicon, diluted 1:500 in PBS containing 20% fetal calf serum, 20% normal goat serum) at 4 °C overnight. Retinas were then washed three times with PBS, 10 min each, incubated with secondary antibody Alexa 594 goat–anti-rabbit (molecular probes; 1:500 dilution prepared in PBS containing 20% FCS, 20% NGS) for 2 h at RT, washed four times with PBS, 30 min each, and mounted on a slide with PBS/glycerol (2 vol/1vol). Retinas were viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Carl Zeiss, Chester, VA). The relative rate of retinal vascular expansion was determined by measuring the radius of expanding retinal blood vessels relative to the radius of the retina from the optic nerve head in P5 PECAM-1+/+ and PECAM-1−/− mice. The central capillary dropout area during OIR was quantified as percentage of whole retina area. The quantitative assessments were made from the digital images in masked fashion using Axiovision software (Carl Zeiss, Chester, VA).

Quantification of neovascular proliferative retinopathy

Quantification of vitreous neovascularization on P17 was performed as previously described (Wang et al., 2003). Briefly, mouse eyes were enucleated, fixed in formalin for 24 h, and embedded in paraffin. Serial sections (6 μm