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Retinal blood vessels develop in response to local VEGF-A signals in the absence of blood flow

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Abstract

The role of hemodynamic forces and other signals from circulating blood in guiding the development of the retinal vasculature was examined by following the growth of these vessels in organ cultures. Retinal vascular development in organ cultures was monitored by immunofluorescent staining of retinal whole-mounts using antibodies against ICAM-2, a specific marker for endothelial cells and by vascular adenosine disphosphatase activity. Under culture conditions, the retinal vasculature from mice at postnatal day 3 (P3) grew from the optic nerve area to the edge of the retina in a manner similar to that observed in vivo. Both inner and outer vascular plexuses formed in retinal explants. Within the first few days of organ culture, the initial uniform meshwork of blood vessels was reorganized into arterioles, venules, and capillaries. As in animals, the initial retinal vascular plexus contained abundant vessels, and afterward some vessels regressed leading to the formation of a mature vascular bed. Changes in vascular density due to blood vessel growth and remodeling were confirmed by RT-PCR and Western blot analyses of ICAM-2 mRNA and protein levels, respectively. In addition, during in vitro retinal vascularization, arterioles acquired mural cell coverage, as shown by positive staining for α-smooth muscle actin. Thus, blood flow and blood-derived signals were not required for the development and maturation of retinal vessels. In contrast, stability of blood vessels in retinal explants was tightly regulated by endogenous levels of vascular endothelial growth factor-A (VEGF-A). VEGF-A was expressed in the explants throughout the culture period, and addition of neutralizing antibodies against VEGF-A to the organ culture caused a severe regression of blood vessels from the vascular front toward the optic nerve. In contrast, addition of anti-FGF-2 antibodies had no effect on the developing vasculature. Thus, retinal vascular development is dependent on local VEGF-A signals rather than systemic signals.
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1. Introduction

The murine retina provides an ideal model to study retinal vascular development and diseases. The process of retinal vascularization in the mouse occurs during the first two weeks of life and is characterized by distinct but overlapping events (Connolly et al., 1988). At birth, the murine retina is largely avascular. Primitive retinal blood vessels, sprouting from vessels in the optic disc, form a uniform meshwork of capillary-like structures immediately surrounding the optic nerve head. By postnatal day 5 (P5), the vascular network has grown to cover approximately 60% of the inner layer of the retina and has been reorganized into a more mature web of vessels, in which arterioles, venules and capillaries can be morphologically distinguished. Around P8, the growth of the superficial, or inner, vascular plexus is complete with the developing blood vessels reaching the edge of the retina (ora serrata). From P4 to P14, blood vessels also grow perpendicularly from the superficial plexus into the inner plexiform layer, and from there extend to the periphery of the retina forming a second vascular plexus (outer or deep retinal plexus), parallel to the first. Between P10 and P21, remodeling events, characterized by regression of surplus segments of capillaries, transform the original vascular web into an essentially mature vascular bed, which is adequate to satisfy the metabolic requirements of the adult retina (Connolly et al., 1988).

There is currently controversy over the mode of formation of the primary (superficial) retinal vasculature. Some investigators propose that vessels of the superficial

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plexus arise by vasculogenesis, the de novo formation of vasculature through differentiation and organization of endothelial cell precursors. Other studies suggest that these vessels arise through angiogenesis, the formation of new blood vessels by migration and proliferation of endothelial cells from pre-existing vessels. This disagreement originates from the use of different markers to identify vascular precursors. However, all investigators agree that the deep vascular bed forms by angiogenesis (Fruttiger, 2002; Gariano et al., 1996; Hughes et al., 2000; Jiang et al., 1995; McLeod et al., 1987; Sandercoe et al., 1999).

At later stages, the newly formed vascular plexus is remodeled to form the mature vascular bed. It has been proposed that vascular segments that are maintained during remodeling depend on blood flow through those segments (Meeson et al., 1996). Alternatively, the delivery of oxygenated blood to the tissues by the newly formed vessels has been suggested to determine ultimate vascular density (Benjamin et al., 1998).

Other studies have shown that both physiological and pathological retinal angiogenesis are under the control of vascular endothelial growth factor-A (VEGF-A) (Provis et al., 1997; Recchia et al., 1998; Stone et al., 1995). VEGF-A is a dimeric glycoprotein that, as a result of alternative splicing of a single gene, exists in a number of isoforms comprising in humans 121, 145, 165, 189 and 206 amino acids (VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206}) (Neufeld et al., 1996; Poltorak et al., 1997). In the mouse three isoforms have been described, VEGF-A_{120}, VEGF-A_{164} and VEGF-A_{188}. Through the interaction with two endothelial cell-specific receptors, VEGFR-1/flt-1 and VEGFR-2/flk-1/KDR (de Vries et al., 1992; Klagsbrun and D'Amore, 1996; Terman et al., 1992), VEGF-A regulates a wide variety of endothelial cell functions, including proliferation, migration, assembly into tubes and survival (Ferrara and Davis-Smyth, 1997). Knockout of VEGF-A or its receptors in mice results in embryonic lethality due to vascular defects (Shalaby et al., 1995). Interestingly, mice lacking a single copy of VEGF-A allele also die as embryos, suggesting that normal blood vessel development requires a specific dosage of VEGF-A (Carmeliet et al., 1996; Ferrara et al., 1996). Recently, distinct roles for the three VEGF-A isoforms in retinal vascular growth and development were revealed in mice generated to express only the single isoforms (Stalmans et al., 2002). In mice expressing only VEGF-A_{164}, retinal vascularization was normal. In contrast, in mice expressing only VEGF-A_{120}, retinal vessel growth was inhibited and development of venules and arterioles was abnormal. In mice expressing only VEGF-A_{188}, normal vascular growth and venular development was observed, but not proper arteriolar formation.

Retinal organ culture systems have been previously used to study photoreceptor development (Soderpalm et al., 1994) and degeneration (Caffe et al., 1993). In these organ cultures, the organization of the three cellular layers (outer nuclear layer including well differentiated rods and cones, inner nuclear layer and ganglion cell layer) with the intervening plexiform layers is maintained and is comparable to that in eyes in vivo (Caffe et al., 1989; Feigenspan et al., 1993; Ogilvie et al., 1999). The overall retinal architecture of the explants is retained for several weeks in culture (Caffe et al., 2001a; Caffe et al., 1989; Sassoe-Pognetto et al., 1996), and normal processes of photoreceptor development and degeneration have been reported to occur (Caffe et al., 2001b; Ogilvie, 2001; Ogilvie et al., 2000). We have examined if these organ cultures can be used to study the events involved in retinal vascular development.

Here we report that retinal explants from newborn mice can be successfully employed to study retinal vascular development. Under culture conditions, the immature vasculature of retinas from newborn mice developed and matured in vitro following a time course similar to that in living animals. In early explants, we observed a rearrangement of the initial undifferentiated vascular network into arterioles, veins and capillaries and their gradual growth across the inner layer of the retina. During culture, blood vessels reached the edge of the inner retina and a deeper (outer) vascular plexus also developed. At later times, extensive remodeling events occurred, leading to the formation of a mature vascular bed. During in vitro development, arterioles acquired a peri-endothelial cell (PEC) coating, as shown by positive staining of cultured retinas with antibodies specific for α-smooth-muscle actin (α-SMA). VEGF-A was expressed in explanted retinas throughout the duration of the organ culture. Addition to the organ culture medium of blocking antibodies against VEGF-A led to a massive regression of blood vessels toward the optic disc, indicating that appropriate levels of endogenous VEGF-A are required for retinal vascular growth and stability in explants. Thus, these experiments indicate that normal retinal vascular development occurs in the absence of blood flow and does not depend on the delivery of oxygenated blood to the retina. Retinal vessels appear to respond primarily to local VEGF-A signals.

2. Materials and methods

2.1. Preparation of retinal explants and antibody treatments

Swiss-Webster mice were obtained from Taconic (Germantown, NY) and bred in house. Animal care was in accordance with institutional guidelines. Three-day old mice (P3) were anesthetized with sodium pentobarbital and the eyes immediately enucleated, placed in sterile PBS and transferred to a laminar flow hood where sterile techniques were maintained for the whole procedure. Under a dissecting microscope, eyes were cleaned of extraneous connective tissue and the anterior chamber (cornea, lens and part of the vitreous) removed from

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