Developmental versus Pathologic Retinal Neovascularization


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Developmental retinal angiogenesis occurs quite late in rodents, starting at postnatal day (P) 0 when vessels emerge from the optic nerve. In situ hybridization using probes for vascular endothelial growth factor receptor (VEGFR) 1 or 2, which label endothelial cells, platelet-derived growth factor receptor (PDGFR) β, which labels pericytes, and PDGFRα, which labels astrocytes, shows astrocytes leading the way while endothelial cells and pericytes follow together. Astrocytes are crucial for retinal vascularization, because they express VEGF and provide a template for vascular development.

In situ hybridization for VEGF shows that it is most strongly expressed by astrocytes in the not-yet-vascularized part of the retina. This part of the retina is hypoxic as revealed by EF5 staining. (EF5, injected in live animals, is reduced by an oxygen-inhibitable reductase resulting in permanent protein adducts that, after tissue fixation, can be stained with an antibody to visualize hypoxic tissue.) The central, vascularized part of the retina displays higher oxygen levels and VEGF mRNA is downregulated in this area. There is a sharp transition from high to low VEGF mRNA at the leading edge of the growing vascular network, which is likely to result in a VEGF protein gradient at this location.

Endothelial cells at the leading edge, so-called tip cells, display a number of features not found on other endothelial cells (stalk cells). They are distinguished by high expression of PDGF-B compared to other endothelial cells (this stimulates pericytes to migrate and cover the growing vessels). Another marker for tip cells is the notch ligand delta like 4 (Dll4). Tip cells also differ in morphology from other endothelial cells. They have long filopodia, similar to growth cones on axons, which might be important in sensing VEGF gradients. The filopodia are eliminated by soluble VEGFR1 suggesting that they are sensitive to VEGF levels. When the eye is injected with VEGF164, flooding all VEGF gradients, filopodia are also disturbed and the expansion of the vascular network is halted.

In summary, during development, negative feedback between VEGF expression and vessel mediated tissue oxygenation leads to a VEGF gradient. Tip cells at the distal end of growing vessels read the gradient and guide vessel growth.

How does this scenario compare to pathologies such as diabetic retinopathy where hypoxia and VEGF expression lead to retinal neovascularization? Oxygen-induced ischemic retinopathy (OIR) in mice can serve as a suitable model to study this. In this model one-week-old mice are exposed to hyperoxia which causes regression of newly developed, VEGF-dependent retinal vessels. This primarily occurs around arteries because oxygen can diffuse
directly through the vessel walls into surrounding tissue and downregulate VEGF in these regions. In the center of the retina around the central hyaloid artery this effect is most pronounced and leads to a large capillary-depleted area. When mice are placed back into normoxia, this capillary-free zone becomes hypoxic, visualized by intense staining with EF5, and increases VEGF production. Two days after return to room air the localized high expression of VEGF in the avascular areas results in initial sprouting from remaining vessels but fails to induce successful revascularization of the capillary-free zones. Instead, vessels start to form neovascular tufts towards the vitreous. Why is the hypoxic, VEGF expressing region not vascularized normally as it occurs during development in the peripheral retina? Could it be that pericytes surrounding new vessels express high levels of VEGF promoting uncontrolled growth of sprouts? This is not what is seen. Instead the greatest VEGF expression is by astrocytes. Astrocytes survive the hyperoxia and hypoxia, although GFAP gets downregulated. The receptors for VEGF are expressed in the endothelial cells in the tufts. Are tip cell markers expressed in the tufts? In situ hybridizations for PDGFB or Dll4 show that they are expressed in tufts, which indicates that tip cells are present, but their location is abnormal in that they are not located adjacent to the avascular areas where the revascularization is supposed to occur. Is there a VEGF gradient? It is very difficult to show at the protein level, but one hypothesis to explain the improper orientation of tip cells in tufts is that the VEGF gradient is disturbed. The following four observations support this view:

1) When mice are placed into 10% oxygen at P4, they become globally hypoxic and there is increased expression of VEGF in the retina. These increased levels of VEGF, which alter the normal gradients, result in vascular growth where it is not usually seen, such as early sprouting downward to form deep capillaries.

2) Mice that are genetically engineered to express only VEGF121 and not the other VEGF isoforms, have reduced survival, but those that survive show neovascular tufts in the retina. These mice have VEGF that cannot localize properly to form normal gradients, and the result is abnormal sprouting to form neovascular tufts.

3) If mice are put in hyperoxia at P7 and kept there instead of being returned to room air at P12, the large avascular areas gradually revascularize without neovascular tufts. While gradients may be flattened in this situation, they are not completely eliminated.

4) Op/op mice have a mutation in colony stimulating factor 1 (CSF-1) resulting in CSF-1 deficiency. This leads to a lack of macrophages. In the OIR model, these mice revascularize the avascular areas without development of neovascular tufts.

A hypothesis that could reconcile these observations is that VEGF gradients are needed for normal vascular development. If the gradients are flattened, vascular development is delayed, but still occurs without abnormal sprouting. But if gradients are severely disturbed or eliminated, then abnormal sprouting occurs. Macrophages contribute to disturbances of gradients in hypoxic retina. In their absence, the increased expression of VEGF by astrocytes and other retinal cells may not disturb the gradient sufficiently to promote abnormal sprouting.

Questions
1. Op/op mice get revascularization without sprouting. Is it possible that in the absence of macrophages there is no degradation of the basal lamina, which allows more rapid revascularization?
The collagen tubes are gone within 48 hours in all mice in the hyperoxia model.

2. Has anyone looked at whether the tip cells are associated with a specific MMP, like MMP9?
Holger Gerhardt has been investigating this and finds that it is not the tip cells that express MMPs, but macrophages. This helps to liberate VEGF from the ECM and may be a mechanism by which macrophages contribute to disturbance of the VEGF gradient.

3. Are expression of PDGF-B andDll4 in tip cells dependent upon each other?
It is not definitely established, but there is some evidence suggesting that PDGF-B is downstream of Dll4.

4. How do tip cells find each other?
They don’t find each other; the stalk cells proliferate and the branching occurs behind the tip cells.