Growth factor gradients in vascular patterning

Andrea Lundkvist, Sunyoung Lee*, Luisa Iruela-Arispe*, Christer Betsholtz† and Holger Gerhardt[1]

*Vascular Biology Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK, *Molecular Biology Institute, Jonsson Comprehensive Cancer Center and Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, 611 Charles E. Young Drive Ea., Los Angeles, CA 99095, USA and †Laboratory of Vascular Biology, Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Scheeles vag 2, Karolinska Institutet, SE-171 77 Stockholm, Sweden*

Abstract. Growth factor gradients regulate many developmental processes. VEGF-A is distributed in a graded fashion in growing tissues in order to direct sprouting of new vessels. Growth factor gradients can be formed by regulated production, retention, controlled release and degradation. VEGF-A production is controlled by hypoxia while its retention depends on the C-terminal heparin-binding motifs present in the longer splice-isoforms, VEGF164 and 188. This motif confers binding to the cell surface and the surrounding extracelluar matrix. The short isoform VEGF120 is diffusible and hence fails to direct endothelial tip cell migration. Conditional inactivation of heparan sulfate proteoglycans in the cells that produce VEGF results similarly in misguidance of the tip cells. Studying retinal developmental angiogenesis and pathological neovascularization side-by-side in the mouse retina, we find that endothelial tip cell guidance and stalk cell proliferation control are disrupted in neovascularization due to a loss of VEGF-A retention. The cause for this is proteolytic cleavage of VEGF-A by matrix metalloproteases (MMP) derived mostly from macrophages infiltrating the ischaemic retinal areas. Genetic or pharmacological inhibition of macrophage infiltration or MMP activity can rescue guided revascularization at the expense of pre-retinal neovascularization. Disruption of VEGF-A gradients provides a novel concept for the mechanism underlying pathological patterning in ocular disease.

2007 Vascular development. Wiley, Chichester (Novartis Foundation Symposium 283) p 194–206

Growth factor and morphogen gradients are versatile tools for patterning previously uniform space into distinct domains, to fine tune cellular responses like differentiation and proliferation and to direct cellular migration (Charron & Tessier-Lavigne 2005). VEGF-A is involved in early differentiation processes of the haemangioblast lineage (Damert et al 2002), but also fulfils numerous func-

[1]This paper was presented at the symposium by Holger Gerhardt, to whom correspondence should be addressed.

tions in subsequent patterning events that lead to the formation of an intricate hierarchical vascular network that is precisely adapted to the various demands of the respective organs (Ferrara et al 2003). The extraordinary dependence of the vascular system on this one growth factor is best illustrated by the fact that both a two-fold reduction (Carmeliet et al 1996, Ferrara et al 1996) and a two-fold increase (Miquerol et al 2000) of VEGF-A levels in mouse embryos is incompatible with survival. Thus VEGF-A levels need to be tightly controlled in the tissue. Additionally, the spatial distribution of VEGF-A appears to be critical for vascular patterning. Our recent studies on the mouse retina and embryonic hindbrain illustrated that a graded distribution of VEGF-A is instrumental in regulating two fundamental processes during sprouting angiogenesis, tip cell migration and stalk cell proliferation (Ruhrberg et al 2002, Gerhardt et al 2003).

The tip cell concept

The leading endothelial cell of the vascular sprout (the tip cell) differs from the following stalk cells in terms of morphology, gene expression and response to VEGF-A (Gerhardt et al 2003, Gerhardt & Betsholtz 2005). The tip cells are induced by high VEGF-A concentrations, they extend multiple long filopodia towards the source of VEGF-A, they migrate towards the VEGF-A source and generate pulling forces, likely through selective adhesion to the provisional matrix by filopodial integrins. They also present the VEGF receptor 2 (VEGFR2) on their filopodia, suggesting a sensor function for these protrusions. The tip cells further express certain genes related to their function as the leading cell. They express PDGF B in order to recruit pericytes to the nascent sprout, and they express Dll4 potentially to repress protrusive activity in the neighbouring stalk cells (see Anne Eichmann's and George Yancopoulos' papers in this book).

Mechanisms of tip cell guidance

Endothelial tip cell guidance bears similarities to guided migration in other organ systems, like axonal guidance during neural development (Carmeliet & Tessier-Lavigne 2005), or sprouting of the *Drosophila* tubular airway system, the trachea (Metzger & Krasnow 1999). Here growth factor gradients, matrix scaffolds and specific guideposts all contribute to guidance of the leading tip structures. Whereas long-range gradients may function to determine the gross direction, matrix scaffolds or guideposts may help to determine a specific trajectory of growth. Interestingly, recent studies have shown that a number of neural guidance molecules and receptors are involved in endothelial tip cell guidance. Thus, attractive growth factor gradients may work in concert with repulsive signals to fine-tune directed migration of the tip cells. However, unlike the growing axon, the multicellular angiogenic sprout requires a co-ordinated migration of the tip and proliferation in the stalk.

Balancing migration and proliferation

The proliferative response in the growing vasculature is largely restricted to the stalk cells in the vicinity of the sprouting front (Gerhardt et al 2003). Their number appears to be controlled by the local levels of VEGF-A. Altering the concentrations and distribution of VEGF-A in the retina by local administration or genetic manipulation allowed us to study the response of tip and stalk cells in this tissue. Based on our observations we proposed that the graded distribution of VEGF-A regulates the balance of tip cell migration and stalk cell proliferation and thereby controls vascular patterning during sprouting angiogenesis.

In the present paper we address the question of how VEGF-A gradients are formed and instruct normal development, and then turn to the model of oxygen-induced retinopathy to ask how pathological neovascularization differs from normal angiogenesis in order to elucidate mechanisms of pathological vascular patterning. We provide the first evidence for a loss of endothelial guidance through disrupted VEGF-A gradients, representing the fundamental cause of retinal vasculopathies (Lundkvist et al 2007, submitted).

Graded versus diffuse VEGF-A distribution

Summarizing previous work, the morphological appearance of a growing vascular sprout differs considerably in situations of graded versus diffuse VEGF-A distribution. Graded VEGF-A distribution leads to rapid tip cell migration and confined stalk cells proliferation only in the vicinity of the VEGF-A source. The resulting sprout is slender with a homogenous stalk diameter and the vascular network is highly branched. The filopodia of the tip cell are polarized, long and confined mostly to the very tip of the sprout. In contrast, diffuse VEGF-A is insufficient to direct tip cell migration but results in widespread stalk cell proliferation.

Mechanisms to form a gradient in the tissue

Gradient formation will depend on the sum of mechanisms that can contribute to raise the concentration at one place above the concentrations in the surroundings. Gradient formation therefore can be regulated at various levels. First of all, the production of the growth factor needs to be regulated in a spatiotemporal fashion that allows higher concentrations to be spatially restricted. VEGF-A production can be induced by many factors in cultured cells. Interestingly, *in vivo*, in the retinal system VEGF-A is largely produced by the astrocytes in the area ahead of the growing vascular plexus (Stone et al 1995, Provis et al 1997, Gerhardt et al 2003). Many studies have shown that hypoxia is the main trigger and regulator for VEGF-

A expression. Oxygenated tissue rapidly shuts down VEGF-A expression (for details on hypoxia regulation see Georg Breier's paper in this book).

Gradient formation also relies on regulated retention of the produced factor. If concentrations are to be maintained at a certain site, say the astrocytes ahead of the vessels, VEGF-A needs to be bound locally in order to maintain high local concentrations. Splicing of the VEGF-A gene into several different isoforms is a powerful tool to regulate retention by including sequences from exon 6 and 7 that code for a conserved stretch of basic amino acids, termed the retention motif. The longer isoforms, 188 and 164 include this retention motif and thus are retained at the cell surface or in the proximal extracellular matrix (Park et al 1993). The cell surface and extracellular matrix is rich in heparan sulfate proteoglycans (HSPGs), which carry sulfated sugar chains that represent ideal attachment sites for basic peptides through charged interactions (Esko & Lindahl 2001). Although the specificity of these interactions is currently debated, it is clear that HSPG are crucial for the formation of many growth factor and morphogen gradients. A recent study by Robinson and Stringer identified parts of the structural requirements for the sulfated sugar chains to effectively bind the VEGF164 dimer (Robinson et al 2006). Our lab has recently turned towards HS function in vascular patterning and begun to study the effect of conditional HS deletion in the various cell-types that interact with the sprouting front. First data on astrocyte specific deletion of HS by GFAP-Cre driven recombination at the EXT1 locus in astrocytes, suggest that astrocytic HS is the key to VEGF-A gradient formation in the retinal tissue. We observed a specific defect in filopodia guidance and stalk cell proliferation control at the leading vascular front, highly reminiscent of the images seen in the VEGF120/120 mice, in which the growth factor lacks the retention motif.

A further mechanism to shape a gradient can be regulated local release and/or degradation. Little is known about these processes in respect to VEGF-A. However, a number of studies have implicated a series of proteases including members of the cathepsin family, matrix metalloproteinases (MMPs), plasmin and others to either alter the binding of VEGF by proteolysis of VEGF itself, or by modifying the binding properties of the matrix (Houck et al 1992, Plouet et al 1997, Lee et al 2005). In fact, it has been suggested that the switch from dormant tumour lesion to highly angiogenic tumour (the so-called 'angiogenic switch') is triggered by mesenchymal MMP production, thus releasing VEGF-A to activate neo-angiogenesis (Bergers et al 2000). Whether local release and degradation is involved in VEGF-A gradient formation in normal development is unclear.

What is the cause of disturbed vascular patterning in pathological neovascularization?

The mouse model of oxygen-induced retinopathy (OIR) closely resembles vascular malformations occurring in situations of ischemia-driven retinal

neovascularization in diabetic patients (Smith et al 1994). Capillary-denuded regions in the retina suffer from tissue hypoxia, triggering a neovascular response. Unlike normal development, this neovascular response fails to re-establish an organized hierarchical pattern, but results in chaotic vascular malformations and hyperproliferation with pre-retinal vessels. These pre-retinal vessels eventually lead to bleeding, fibrosis and detachment. From a therapeutic perspective, two separate aspects require attention: some areas have too few vessels, whereas other areas develop too many and in ectopic positions. Thus the ideal therapeutic approach should work towards guiding effective vessel regrowth into the avascular region while inhibiting formation of pre-retinal vascularization.

In the mouse model of OIR, the primary retinal plexus forms normally in the first postnatal week P1–P7. Subsequently, the pups are housed in 75% oxygen, leading to regression of capillaries in the central regions. On day P12, the pups are returned to room air oxygen levels (21%) for another 5 days (Smith et al 1994). During this phase, the neovascularization is triggered leading to pre-retinal vascular tufts, hyperproliferation of the veins and arterial tortuousity. Examining proliferation by BrdU injection and tip cell polarization by lectin immunofloures-cence and filopodia measurement (Gerhardt et al 2003), we observed that the onset of neovascularization closely resembled organized patterning similar to normal developmental angiogenesis. Endothelial cells only proliferated in the vicinity of the leading sprouting front and tip cells extended long filopodia along VEGF-producing astrocytes. This organized pattern of proliferation and filopodia extension was disrupted on days 13–17. The resemblance of the vascular phenotype during retinal neovascularization to observations on experimental disruption of VEGF-A gradients, led us to ask whether VEGF-A gradients may be disturbed in OIR. In an attempt to test this, we combined VEGF IHC with VEGF ISH and BrdU labelling to determine the spatial relationship between the site of VEGF-A production, the site of VEGF-A protein localization, and the site of the endothelial response. At day 12, VEGF-A production at the mRNA level was confined to astrocytes in the avascular zone and absent around the arteries. VEGF-A protein was also absent around arteries and arterial ECs did not proliferate. However, at day 17, arterial ECs readily proliferated, and VEGF-A protein was also detected in the vicinity of these proliferating cells. Interestingly, *in situ* hybridization revealed that VEGF-A mRNA was still absent around arteries, suggesting that VEGF-A protein must have relocated (diffused) away from the producing cells. We performed RT-PCR to determine whether this could be explained by up-regulation of the non-heparin binding isoform VEGF120, but found that the heparin binding isoform VEGF164 was predominantly up-regulated at mRNA levels. This suggested that VEGF-A relocalization was not caused by an isoform switch. A recent study showed that a novel truncated form of VEGF-A is produced by proteolytic cleavage at the C-terminal end by members of the MMP family (Lee et al 2005).

The resulting proteolytic fragment VEGF113 lacks the heparin binding C-terminus, but contains the receptor binding domains. Using a sandwich ELISA assay with two different antibodies that recognize the N-terminus and the C-terminus of VEGF-A, we determined the ratio of cleaved versus uncleaved VEGF in retinal samples from normal retinal development, OIR at P12 and P14 as well as P17. We also included control samples from mice exposed to continued hyperoxia, in which revascularization of the retina occurs without pathological pre-retinal vessels (Gu et al 2002). The samples from normal developmental stages, P12 and continued hyperoxia controls, showed no detectable levels of cleaved VEGF-A, suggesting that this MMP-dependent processing of VEGF-A does not contribute to normal development. However, in OIR at P14 and increasingly at P17, cleaved VEGF-A constituted up to 80% of the total VEGF-A. Thus, more than half of the VEGF-A present in OIR lacked the heparin-binding C-terminus and consequently would lack retention at the cell surface or in the surrounding extracellular matrix. In comparison, mice that have one VEGF-A allele replaced with the VEGF120 isoform develop similar pre-retinal neovascularization during normal development, arguing that even 50% of diffusible VEGF-A is detrimental for guided vascular growth.

We next asked whether MMPs are involved in this cleavage *in vivo* and therefore tested a broad-range inhibitor of MMPs (GM6001) by i.p. injection during the neovascularization period. Interestingly, we observed restored re-vascularization and normalized patterning concomitant with polarized filopodia protrusion and restored proliferation control. Furthermore the VEGF-A protein localization was normalized in these retinas, arguing that indeed MMPs are involved in the VEGF-A redistribution in the pathological phase of OIR. In a gain-of-function approach we asked whether local administration of recombinant MMPs to a normal unchallenged retina would mimic a loss of VEGF-A gradients. 24 h after intravitreal delivery of MMP9, MMP3 or MMP12, we observed substantial shortening of tip cell filopodia and a hyperplasia of the stalks reminiscent of VEGF-A injection. Injection of pro-MMP9 had no effect. After 48 h, on P7, pre-retinal vascularization occurred, suggesting that these MMPs are sufficient to cause vascular malformations similar to OIR. RT-PCR for all MMPs and their natural inhibitors (TIMPs) surprisingly showed prominent up-regulation of only one member, MMP12. *In vitro* MMP12 potently cleaved VEGF-A, while addition of GM6001 completely abolished VEGF-A cleavage. MMP12, also known as macrophage elastase, is specifically expressed by macrophages. In OIR, macrophages are most abundant in the capillary-free, hypoxic areas. This recruitment did not occur in CSF-1op/op mice and could also be inhibited by clodronate-liposome administration i.p. and intravitreally (Van Rooijen & Sanders 1994). Inhibition of macrophage recruitment led to a similarly normalized re-vascularization as found after GM6001 treatment. We tested the potential involvement of MMP12 by analysing MMP12-deficient

mice (Shipley et al 1996). MMP12-deficient animals showed no alterations in any aspect of normal retinal angiogenesis and also showed a similar vascular regression following the hyperoxia treatment. However, already heterozygous animals showed significantly improved re-vascularization and reduced epiretinal tuft area following OIR. We conclude that MMPs are necessary and sufficient to disrupt VEGF-A localization, which is otherwise dependent on C-terminal interaction with heparan sulfate.

Conclusions

Normal vascular patterning depends on VEGF-A gradients formed through localized production and retention of the longer VEGF-A isoforms 164 and 188. In the OIR model of pathological vascular patterning, VEGF164 is cleaved by MMPs resulting in a loss of retention and directed re-vascularization. Inhibition of MMP-dependent VEGF-A cleavage is capable of restoring VEGF-A distribution, and therefore restoring guided re-vascularization and functional vascular patterning. Our present results further highlight the involvement of macrophages in OIR. For human complications such as diabetic retinopathy, the involvement of MMPs has been suggested, however, we provide evidence for a novel mechanism by which MMPs may lead to pathological vascular patterning. The wide range of MMPs that have the capability of cleaving VEGF-A however suggests that identification of one definitive member may be difficult and several different MMPs may be involved.

Acknowledgements

This work was supported by Cancer Research UK. We are grateful to Lieve Moons and Peter Carmeliet for providing MMP-9 and MMP-12 deficient animals for this study. Thanks to Marcus Fruttiger for valuable comments and discussions.

References

Bergers G, Brekken R, McMahon G et al 2000 Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2:737–744

Carmeliet P, Tessier-Lavigne M 2005 Common mechanisms of nerve and blood vessel wiring. Nature 436:193–200

Carmeliet P, Ferreira V, Breier G et al 1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439

Charron F, Tessier-Lavigne M 2005 Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development 132:2251–2262

Damert A, Miquerol L, Gertsenstein M, Risau W, Nagy A 2002 Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation. Development 129:1881–1892

Esko JD, Lindahl U 2001 Molecular diversity of heparan sulfate. J Clin Invest 108:169–173

Ferrara N, Carver-Moore K, Chen H et al 1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442

Ferrara N, Gerber HP, LeCouter J 2003 The biology of VEGF and its receptors. Nat Med 9:669–676

Gerhardt H, Betsholtz C 2005 How do endothelial cells orientate? EXS 3–15
Gerhardt H, Golding M, Fruttiger M et al 2003 VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161:1163–1177
Gu X, Samuel S, El-Shabrawey M et al 2002 Effects of sustained hyperoxia on revascularization in experimental retinopathy of prematurity. Invest Ophthalmol Vis Sci 43:496–502
Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N 1992 Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 267:26031–26037
Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML 2005 Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol 169:681–691
Lubarsky B, Krasnow MA 2003 Tube morphogenesis: making and shaping biological tubes. Cell 112:19–28
Metzger RJ, Krasnow MA 1999 Genetic control of branching morphogenesis. Science 284:1635–1639
Miquerol L, Langille BL, Nagy A 2000. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. Development 127: 3941–3946
Park JE, Keller GA, Ferrara N 1993 The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 4:1317–1326
Plouet J, Moro F, Bertagnolli S et al 1997 Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. J Biol Chem 272:13390–13396
Provis JM, Leech J, Diaz CM, Penfold PL, Stone J, Keshet E 1997 Development of the human retinal vasculature: cellular relations and VEGF expression. Exp Eye Res 65:555–568
Robinson CJ, Mulloy B, Gallagher JT, Stringer SE 2006 VEGF165-binding sites within heparan sulfate encompass two highly sulfated domains and can be liberated by K5 lyase. J Biol Chem 281:1731–1740
Ruhrberg C, Gerhardt H, Golding M et al 2002 Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16:2684–2698
Shipley JM, Wesselschmidt RL, Kobayashi DK, Ley TJ, Shapiro SD 1996 Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. Proc Natl Acad Sci USA 93:3942–3946
Smith LE, Wesolowski E, McLellan A et al 1994 Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci 35:101–111
Stone J, Itin A, Alon T et al 1995 Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J Neurosci 15:4738–4747
Van Rooijen N, Sanders A 1994 Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 174:83–93

DISCUSSION

Drake: Your work shows that tip cells send out multiple processes. VEGF appears to be promoting this huge exploratory front, as if the cell is not sure where to go rather than following a tight gradient. When we watch the expanding vascular front in allantois cultures (which look like your retinal network), there are

leading cells and they send out numerous filopodia. Based on the predictable vascular pattern that is formed, one can tell which filopodial connections will lead to new blood vessels and form the next part of the network. It seems that what VEGF is doing at the tip is promoting the formation of numerous filopodia, so the gradient is rather more like a general stimulant. What do you believe mediates the persistence of one filopodia versus another?

Betsholtz: Are you asking how the filopodia that contact those of the other cell are chosen, or how the cell extending the filopodia was chosen in the first place?

Drake: The tip cell's business is sending out the filopodia, which is the working end of the tip cell. Do you think there is a broad gradient of VEGF?

Gerhardt: In the retinal model it is clear that there isn't a broad gradient. VEGF is definitely stimulating this whole process; if we inhibit VEGF we can show that the protrusive activity is a behaviour dependent on VEGF. The VEGF distribution can be altered in the retinal system by putting animals into hyperoxic or hypoxic conditions. This changes the levels of VEGF that are produced within the plexus (vascularized retina) relative to ahead of the plexus (avascular retinal periphery). Ahead of the plexus where no vessels have yet formed, the level of oxygen that the animal breathes has no effect. It is the balance between VEGF ahead and behind the leading front that is shifted by tweaking the oxygen concentration the animal breathes. By doing this you can fine tune how rapidly the retinal vasculature grows out, and how extensively stalk cells proliferate. In hypoxic conditions there is more VEGF in the already vascularized area, thus reducing the spatial differences in VEGF levels between avascular periphery and vascularized retinal centre. In many cases, these relative differences are tightly controlling the response.

If MMPs are inhibited during normal development, nothing happens. We don't think that MMPs are doing the same job in normal development as in pathological neovascularization. In fact, it is unclear whether they have any role in normal vascular development. The macrophages that I presented as a key to pathological neovascularization seem to have a different role in development. Retinas that lack macrophages have even more extreme directionality of the tip cell response. In these retinas, tip cells show less explorative behaviour, leading to less branching, but the migration of the tip cells is not affected at all. The tip cells run along exactly the direction that you'd predict because of the VEGF gradient.

With regard to the question of fusion, or what determines which cells will finally connect, we have made an interesting observation: in the absence of macrophages, endothelial tip cells are not mutually attracting each other. The fusion points coincide with macrophages in almost all tissues in the mouse embryo. In zebrafish, we have been looking at the dynamics of this behaviour. The macrophages whiz around the tip cell. When they do this, the explorative behaviour of the tip cell is dramatically increased. At the fusion points, however, the macrophages appear rather immobile and sit there until the fusion is completed.

Drake: Perhaps there is an unappreciated role for macrophages during vasculogenesis.

Betsholtz: I am not sure that filopodia guidance is the same as cell guidance here. It would be interesting to hear comments from someone more knowledgeable than me about this. I am not convinced that filopodia are at all guided towards the VEGF gradient. Instead they might be selectively stabilized through VEGF signalling. The initial response to VEGF might be broad filopodial protrusion (which can be modulated by macrophages). Subsequently selective stabilization may occur of those filopodia that sense the highest VEGF concentration, or attach to macrophages, or sense soluble macrophage-derived cues. The stabilized filopodia may point out the direction of the cell movement.

Ruhrberg: I liked your description of potential mechanisms that may operate during endothelial cell guidance, i.e. the gradient, the matrix scaffolds and the guide post cells. These mechanisms may not be mutually exclusive, and, in fact, the retina work shows that they are not mutually excusive. You have the VEGF gradients, but you also have matrix scaffolds, as you showed how the astrocyte network prefigures the patterning of the overlying vessels. In your pictures at high magnifications, this co-patterning is seen already at the level of endothelial cell filopodia and astrocyte extensions. So it seems that there is a general growth factor gradient that attracts the vessels and also a scaffold for vessel filopodia to track on. It is probably the intersection of these two different pathways that achieves the final pattern, i.e. there is directionality provided by the gradient and fine patterning due to the shape of the astrocyte scaffolds.

Gerhardt: We have been studying these matrix scaffolds in more detail. One student in my lab has done extensive work looking at all the different laminin forms, and fibronectin, in the context of the astrocytes as well as along the growing tip. A couple of laminins are consistently expressed by the astrocytes and fibronectin is also produced by the astrocytes. Interestingly, if we knock out fibronectin specifically in the astrocytes, we don't see a lack of filopodial guidance. It only impacts on the speed of the tip cell migration. The filopodia still follow the astrocytes.

Drake: During vasculogenesis, we also see fibronectin- as well as integrin α5β1-mediated regulation of tip cell behaviour.

Gerhardt: It may depend on the tissue context. We are in the process of deleting the other laminins in retinal astrocytes. They produce a complex matrix. We also have data showing that heparan sulfate proteoglycans are produced by the same cells. Our data do suggest that VEGF gradients are indeed involved in guiding filopodia. Most importantly, there is a massive misguidance phenotype in VEGF120 mice. It looks like a matrix-bound form of VEGF is involved in this guidance. But you are comparing it to stabilization and we may have a combination of both.

Betsholtz: I am trying to reconcile this with Brant Weinstein's videos of tip cells spearheading the intersegmental vessels in zebrafish embryos. There is apparently

extensive dynamic explorative behaviour, with filopodia extending towards or even into the somites, followed by their retraction. I don't think the filopodia are guided when they go off the track, it is just that they are not becoming stabilized. The net outcome is the guided cell migration, but the filopodial protrusions by themselves might be more random and part of a generic machinery triggered by VEGF.

Weinstein: That is basically the way that neuronal guidance works. There aren't selective protrusions. Protrusion is a fairly dynamic and stochastic process, but it is the stabilization of filopodia going the right way or repulsion of filopodia that go the wrong way that matters.

Gerhardt: I agree. When looking at these analogies, I would have thought this is what is happening in our model as well. However, the surprising finding about the macrophages is that when they are gone, the filopodia appear not to be extending in the same stochastic way. They appear to be much more confined and directional. What are the macrophages doing? They seem to be binding VEGF very strongly. We are currently studying how they may cause this intensive explorative behaviour in the tip cells. The macrophages appear to bind up VEGF while sitting together with the astrocytes and could be affecting filopodia by releasing it again. Localized and matrix bound VEGF could potentially aid in stabilizing the filopodia.

Weinstein: If you look at a static picture and it looks like there are more filopodia heading in the direction of the macrophage, you could be fooled: you might not be seeing the finest ones but just the largest ones that have become stabilized, and you have to look at the dynamic. How many protrusions per minute are going in a certain direction?

Gerhardt: This is exactly why we turned to the zebrafish model. It looks very different, even if you take still pictures of zebrafish intersegmental vessels they look different from what is seen in the retina. This indicates that perhaps there are some aspects that are not quite the same, with more polarization in one system than the other. When we looked at particular issues of the dynamics, it seems stochastic in many areas. In the context of macrophages there are interesting differences, where the protrusive activity goes towards the macrophage and collapses when the macrophage disappears.

Lammert: I want to ask about the tube mechanism. When the tip cells fuse to connect the vessels, are they seamless? Are the stalks seamless without junctions? Where is the basal lamina?

Gerhardt: We don't know yet whether the exact fusion point is seamless. However, we have looked at the electron microscopic level on ultrathin sections of the retina and found nice tip cells and filopodia in contact with the astrocytes. This allows us to look at the junctions, the lumen and matrix deposition. Interestingly, first of all we didn't find any vesicles in the retinal system. We didn't find any lumenal matrix. But to our surprise, we found formations of basal membrane already on the filopodia. We also found that the tip cell itself produces specific laminins. It

seems that there's something else to look at in terms of what the tip cell does. I would like to get away from the dogma that the sprout needs first of all to degrade a lot of matrix. For a developmental system it might be different; if you want to invade into a scar tissue degrading matrix might be more important.

Lammert: Are the stalks seamless or do they have autocellular junctions?

Gerhardt: They have autocellular junctions. But I think there may be situations where one cell or two cells are found. They are definitely not seamless.

Ye: I am curious why the new vessel always grows toward the inner limiting membrane and never into the ganglion cell layer.

Gerhardt: I think this has something to do with the particular properties of the retina.

Ye: What happens if you inject VEGF into the vitreous?

Gerhardt: If you inject VEGF, you get these kinds of problems, as you do if you express VEGF from the lens. But also if you just have endogenous cells expressing only diffusible VEGF, then all of a sudden the vessels grow into the vitreous. This indicates that the loss of guided and directional growth into the deeper retinal layers, is what really causes the problem of growing up into the vitreous. This is probably the reason why the retinal vasculature is so sensitive to malformation. The vessels are in very close contact with this inner retinal surface, and once they make it into the vitreous, things go terribly wrong. In normal development, small bursts of filopodia are also protruding towards the inner limiting membrane at the very same site where new sprouts are forming to grow into the deeper retinal layers. This indicates that perhaps the initial activation of the vessel to form a new sprout is not a directed response. Then you need the gradient to guide the sprout down. If this doesn't occur they just make it through to the vitreous.

Dejana: As soon as the filopodia touch other cells they stabilize. The VE-cadherin will be concentrated at that point. There should be a role for these junctional proteins in stabilization of the cells. What happens if some of these junctional proteins are missing?

Gerhardt: We have some preliminary data using the VE-cadherin blocking antibody where we find that in the deeper plexus, when we inject into the retina, the tip cells sprout towards each other but they fail to make contact. This would be something that would be much better to examine in a dynamic fashion. If we look at dynamics, it does not look as if the moment a tip cell makes contact with another tip cell, they immediately stabilize contact and start to fuse; there is quite a long interaction.

Weinstein: That is not entirely true. In many cases we see behaviour where a vessel has a guided track along which it is supposed to reach and fuse with another vessel, and we see dynamic activity not only in the vessel that is extending and reaching for the point of contact, but also in the vessel that it is going to be contacting. As soon as you make contact all that activity stops.

Gerhardt: We've seen the same, but it is not the first filopodia that meet by chance that will immediately lead to stabilization. There can be some time.

Weinstein: But not much. Once it makes contact it is pretty quick.

Drake: In our system we see filopodia that make contacts that are clearly not in the right position, based on the pattern of vessels that is generated. These contacts do not appear to lead to the formation of a blood vessel. I don't know how a particular contact is chosen, but there are many sprouts and connections that do not persist.

Dejana: In culture, when two cells meet there is a repeated touching back and forth and then they establish a junction.

Owens: It may be that both mechanisms are operative: perhaps a key distinction we need to make is whether it is a 'genetically programmed' vascular network versus a searching/probing activity that is more generic for a remodelling network. Brant Weinstein, I believe you suggested that in the former case you have two cells that 'know' they have to join, and as soon as they touch they cement that juncture. In contrast, with the tip cell probing we are seeing in these diffuse gradients, they are responding to a lot of gradients and the presence or absence of macrophages. These contacts may be much more transient than fixed depending on the local environmental cues.

Weinstein: It could also be a time scale issue. Many cell biologists look at things in time scales of seconds. At that time scale it may look like there is a lot of touching or feeling or exploring. A lot of what we do is looking at minutes or hour time scales, where behaviour seems to be much more rapid and directed.

Gerhardt: Our videos were shorter. We are probably talking about different time scales.

Shibuya: I am interested in macrophages. In this situation we have up-regulation of VEGF. So, there are two possibilities. One is that inflammatory cytokines such as interleukin (IL)6 recruit macrophages, or another is that up-regulated VEGF stimulates VEGFRs on the macrophage to recruit the cell. Which is the major player?

Gerhardt: This is exactly what we need to look at: how much of this macrophage infiltration is due to VEGF? The data from Dave Shima and Tony Adamis suggest that VEGF164 is a very strong proinflammatory agent, and in this retinopathy model it may actually be VEGF that is *the* inflammatory agent. Published data show that other factors are also involved. For example, if MCP1 is inhibited, this reduces the recruitment of macrophages but not to full extent. There might be several factors working together, but this is not surprising.

Shibuya: Is this kind of macrophage-involvement almost always seen in pathological retinopathies?

Gerhardt: There are data suggesting that macrophages contribute to the problem in other retinopathies including choroidal neovascularization in the context of age-related macular degeneration. It is probably true for most retinal vasculopathies.