Retinal and Choroidal Angiogenesis

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Edited by

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PREFACE

Eye diseases with retinal or choroidal angiogenesis as a critical pathological feature are responsible for the majority of all cases of blindness in developed countries. Thus, due to its profound impact, ocular angiogenesis is an intensely studied process, and the field is advancing at an astounding pace. The growing number of investigators interested in ocular angiogenesis has compounded the increasingly difficult task of managing all of the available information. We, therefore, thought that it was time to take stock of the collective research, to focus on its important and potentially beneficial aspects, and to summarize the progress to date.

The contents of this book are based on the proceedings of the Retinal and Choroidal Angiogenesis Symposium, held at Vanderbilt University on October 15 and 16, 2004. The Symposium was generously sponsored by the National Eye Institute and a number of interested pharmaceutical companies, mentioned below. The primary goal of the Symposium was to promote the exchange of current information and ideas among basic and clinical scientists. It was our intention to foster a better understanding of the basic mechanisms underlying ocular angiogenesis and to advance the development of therapeutic interventions. To this end, we featured a collection of investigators from diverse research and clinical centers throughout the United States, ranging from cell and developmental biologists to clinician-scientists. Specifically, we wished to address three aims: (1) to facilitate scientific exchange and collaborative interaction among senior investigators in the field; (2) to create an opportunity for students, young researchers, and fellows to meet and interact with established investigators; and (3) to provide the impetus for this published work.

This book encompasses a broad spectrum of topics related to angiogenesis within the eye. Topics include basic information on the cellular and molecular mechanisms of retinal and choroidal angiogenesis, animal models of ocular angiogenic conditions, novel therapeutic strategies for the treatment of these conditions, drug development efforts to address these novel strategies, and the application of new mechanistic theories to human disease pathogenesis. The book seeks to emphasize basic principles rather than specific experimental results, although contributors were encouraged to use recently acquired data to illustrate points of broader theoretical significance. I have attempted to arrange the chapters and their topics so that a progression exists, beginning with a description of research tools, model systems, and an examination of the molecular facets of the angiogenic cascade, and ending with the most recent efforts to translate these facets into molecular targets for drug development efforts.

The target audience is the interested professional – basic scientist, clinician-scientist, or physician – whether involved in the field of ophthalmology or in other disciplines in which angiogenesis is important. That such a spectrum of topics on such a complicated subject could be encompassed in a single book may seem a daunting goal. Yet, I believe that we have met it. This is a tribute to the contributors' command of their subjects, their range of interest, and the energy and enthusiasm that they brought to the task. And, it is clearly evident as one reads the chapters.

I would like to thank all those who have participated as speakers and as authors. Without their willingness to attend the Symposium and to meet submission deadlines for their contributions, this book would not have been possible. Neither would it have been possible without the help, support, and encouragement of several others: Paul Sternberg, Jr., M.D., the chair of Ophthalmology and Visual Sciences at Vanderbilt University, who provided valuable advice and Department funding to get the project started; Melissa Stauffer, Ph.D., at Scientific Editing Solutions, who spent many hours poring over the chapters and providing other services related to the editing process; Yolanda Miller, who provided on-site support to the participants and attendees of the Symposium; Peter A. Dudley, Ph.D., of the National Eye Institute, who offered a number of suggestions that improved the Symposium and helped us to meet our aims; and finally, Kathy Haddix, who handled communication with Symposium participants, made sure that they were comfortable while in Nashville, and planned and hosted the meals and social functions. Her efforts were tireless and her positive influence was felt by every participant and attendee. The Symposium also received generous financial support from Pfizer Global, Alcon Laboratories, Eyetech Pharmaceuticals, and Genentech. In addition, I would like to thank the local

attendees, faculty and students alike, who were present at the Symposium and who asked terrific questions and stimulated excellent discussion.

It is my sincere hope that this volume will be useful as an introduction to angiogenesis in the posterior segment of the eye, and as a reference source for both established researchers and novices in the field.

John S. Penn

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2. Stanley J. Wiegand
3. Karl G. Csaky
4. Gerard A. Lutty
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8. David P. Bingaman
9. Jonathan M. Holmes
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11. Azza El-Remessy
12. Dale L. Phelps
13. Robert N. Frank
14. Bruce A. Berkowitz
15. Lois E. H. Smith
16. George L. King
17. John S. Penn
18. Asher Weiner
19. Martin Friedlander
20. Janet C. Blanks
21. Maria B. Grant
22. S. Patricia Becerra
23. Ruth B. Caldwell
24. Tailoi Chan-Ling

INTRODUCTION

I am honored to be asked to write the introduction to this book, *Retinal and Choroidal Angiogenesis*. Its publication is very timely because of rapid progress in the treatment of neovascular age-related macular degeneration by FDA-approved angiogenesis inhibitors[1,2] and because of the initiation of clinical trials of this therapy for other types of ocular neovascularization. Professor Penn has organized a comprehensive and forward-looking set of central issues that inform the molecular basis of ocular neovascularization and its modern therapy. He has invited a distinguished group of authors to discuss these topics and to think about future directions.

Taken together, the chapters in this book reveal certain principles of ocular angiogenesis that have emerged from the study of tumor angiogenesis. Endogenous inhibitors of angiogenesis are expressed by different types of cells in the eye and are stored in different matrix compartments. These inhibitors counterbalance pro-angiogenic molecules in the eye. Most neovascular diseases of the eye begin with a shift of the angiogenic balance to the pro-angiogenic phenotype, termed the "angiogenic switch" in cancer biology.[3,4] Increased expression or mobilization of pro-angiogenic proteins, accompanied by decreased expression or deficiency of anti-angiogenic proteins, can be mediated or potentiated by hypoxia, infiltration of inflammatory cells or immune cells, accumulation of platelets and bone marrow-derived endothelial cells at an angiogenic site, changes in stromal fibroblast expression of angiogenesis inhibitors, and other events.

A recent significant advance in treating diseases of ocular neovascularization by anti-angiogenic therapy is based on the development of drugs that neutralize a pro-angiogenic protein, vascular endothelial growth factor (VEGF). However, it took more than four decades for this

angiogenic molecule to be identified and characterized as a target for anti-angiogenic therapy of the eye. The journey was circuitous. In 1945, Algire *et al*. suggested that a diffusible "factor" could mediate tumor neovascularization.[5] In 1948, Michaelson suggested that a diffusible "X-factor" could mediate neovascular retinopathies.[6] Similar proposals of the existence of diffusible angiogenic factors were reported by others from experiments with tumors implanted in the anterior chamber of the guinea pig eye[7] and from transfilter diffusion studies of tumors in the hamster check pouch.[8,9] However, none of these experiments yielded a purified angiogenic molecule.

In fact, efforts to completely purify a tumor-derived angiogenic factor were driven by a hypothesis that I published in 1971 that tumor growth is angiogenesis-dependent.[10] This report also proposed that "anti-angiogenesis" could be a new therapeutic principle for cancer. This paper predicted the future discovery of angiogenesis inhibitors and that neutralization of a "tumor angiogenic factor" by an antibody could be therapeutic. Accordingly, we began to purify angiogenic activity from tumor extracts and to develop bioassays for angiogenesis.[11] These bioassays included the implantation of tumors into a corneal micropocket in experimental animals[12] and the development of sustained-release polymers that could be implanted into the corneal pocket to quantify the angiogenic activity of tumor-derived proteins.[13] Throughout the 1970's this hypothesis was widely ridiculed. However, when removal of pro-angiogenic sustained-release pellets from corneas was followed by complete regression of the induced neovascularization,[14] our confidence was boosted, and we persisted in the purification of an angiogenic factor from a tumor. Since then, experimental ocular neovascularization has been essential for continued progress in the field of angiogenesis research.

In 1984, we reported the complete purification by heparin-affinity chromatography of a capillary endothelial growth factor isolated from a tumor,[15] and in 1985 its angiogenic activity.[16] Subsequently, Esch *et al*. determined the amino acid sequence of a pituitary-derived protein, basic fibroblast growth factor (bFGF),[17] previously isolated and partially purified from brain tissue by Gospodarowicz.[18] In 1986, Klagsbrun in our laboratory determined that our capillary endothelial growth factor had the same sequence as bFGF.[19] In 1983, Senger in Harold Dvorak's lab reported that tumor cells secreted a vascular permeability factor (VPF), which promoted ascites.[20] It was not known to be an endothelial growth factor at that time. By 1989, Rosalind Rosenthal in my laboratory employed heparin-affinity chromatography to purify to homogeneity a second endothelial growth factor. She had isolated this protein from sarcoma 180 cells, and it was not bFGF. We had set out to make sufficient quantities of the protein to

determine its amino acid sequence when I received a call from Napoleone Ferrara of Genentech. He had heard that we had purified a new endothelial mitogen from a tumor. He had also purified a new endothelial mitogen from pituitary cells. He had already determined the amino acid sequence of his protein and offered to sequence our protein for comparison. This was opportune for us, because we faced at least another year of work to produce sufficient protein to sequence it ourselves. Ferrara determined the amino acid sequence of our protein and found it to be identical to his. Ferrara's paper on this second endothelial growth factor appeared in 1989, entitled "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells."[21] Our paper appeared in 1990, entitled "Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor" (VEGF).[22] VPF also turned out to be identical to VEGF. Ferrara and Henzel were our co-authors because they had determined the amino acid sequence for us. I have outlined this history in more detail than is customary for an Introduction, because it reveals the importance of heparin affinity in purifying angiogenic proteins, and because it was the prelude to future collaborations between Ferrara's lab and mine.

We reported the first angiogenesis inhibitor in 1980 using the same bioassays, and reported eleven others over the next 25 years.[2] Eight of these were endogenous angiogenesis inhibitors, including angiostatin and endostatin.

However, by the early 1990's, it was still not clear how retinal angiogenesis or other ocular neovascular pathologies were mediated. Was Michaelson's "X-factor" bFGF, VEGF, or a different endothelial mitogen? Anthony Adamis in my laboratory began a formal exploration of this question.

By 1993, Adamis *et al*. could report that human retinal pigment epithelial cells secreted VEGF.[23] Patricia D'Amore, a co-author on this paper, was also a post-doctoral fellow in my lab. She is currently Professor of Ophthalmology at Harvard (Schepens Institute). During this period, Adamis also carried out the experiments that led to the development of pegaptanib (Macugen). In 1994, in collaboration with Joan Miller at the Massachusetts Eye and Ear Infirmary and Harold Dvorak at the Beth Israel Hospital, we reported that VEGF was significantly increased in the vitreous of monkey eyes when retinal neovascularization was induced by laser injury.[24] We also reported at that time that samples of human vitreous obtained from diabetic eyes revealed very high levels of VEGF.[25] In the same year, Lloyd Paul Aiello at the Joslin Clinic also reported high levels of VEGF in diabetic vitreous.[26] By the following year, in collaboration with Napoleone Ferrara of Genentech, we had demonstrated that retinal cells subjected to hypoxia significantly increased their expression of VEGF, and that VEGF was the

primary endothelial cell mitogen made by those cells.[27] By 1996, we showed that iris neovascularization associated with retinal ischemia in monkeys was prevented by treatment with an antibody to VEGF, the precursor to bevacizumab (Avastin), given to us by Napoleone Ferrara.[28] This became a seminal paper, because it proved that an antibody to VEGF could be used as a drug to treat ocular neovascularization in a non-human primate. It became the basis for (i) other experimental models of therapy of retinal neovascularization,[29] (ii) anti-angiogenic therapy of human neovascular age-related macular degeneration,[30] and (iii) clinical trials of anti-angiogenic therapy for diabetic retinopathy. In December 2004, Macugen was approved by the FDA to treat neovascular age-related macular degeneration, and in June 2006, ranibizumab (Lucentis) was also approved for this indication.

FUTURE DIRECTIONS

Long-term maintenance of angiostatic therapy. A recent report reveals that intravitreal endostatin is effective in treating experimental choroidal neovascularization in mice.[31] These experiments suggest that endostatin may be used to treat neovascular age-related macular degeneration, analogous to its use as a "replacement therapy" in experimental atherosclerosis.[32,33] Endostatin suppresses endothelial responsiveness to a wide spectrum of pro-angiogenic stimuli in pathological neovascularization,[34,35] but not in reproduction or wound healing. Endostatin has shown no side effects in animals or during clinical trials. Therefore, it may also be useful for long-term "maintenance" therapy for patients with ocular neovascularization whose sight has been restored by intravitreal ranibizumab or bevacizumab. Endostatin could be administered subcutaneously or by intravitreal injection.

Angiogenesis-based biomarkers in urine and blood. In the future, anti-angiogenic maintenance therapy of ocular neovascularization could possibly be monitored by quantification of metalloproteinases in urine[36] or by analysis of the platelet angiogenesis proteome.[37,38] Microscopic tumors in mice can be detected by analysis of the platelet angiogenesis proteome because platelets sequester and accumulate VEGF and other angiogenesis regulatory proteins that these tumors release. It is possible that analysis of the platelet angiogenesis proteome could also be used to detect recurrence of choroidal vascular leakage or to detect an increase in choroidal neovascularization, long before detection by ophthalmoscopy. In other words, "ultra-early" prediction of patients at risk for ocular neovascularization may eventually be possible by quantification of angiogenesis-based biomarkers in blood or urine.

Betacellulin. Early clinical trials of ranibizumab in diabetic retinopathy reveal that visual acuity can be improved, but that higher doses, or more frequent dosing, may be required than are currently used for macular degeneration. It is possible that in addition to VEGF, there is another mediator(s) of angiogenesis in the diabetic retina, for example, betacellulin. We first isolated, purified, and determined the amino acid sequence of betacellulin from conditioned medium of proliferating neoplastic beta cells of murine pancreatic islets.[39] Betacellulin is a 32-kD new member of the epidermal growth factor family with 50% homology to TGF-alpha. It is a mitogen for retinal pigment epithelial cells and for smooth muscle cells. We hypothesized that "regenerating beta cells in the diabetic pancreas may release excessive amounts of betacellulin."[40] Retinal pigment epithelial cells contain high concentrations of bFGF, which is a potent angiogenic peptide. Stimulation of retinal pigment epithelial cells by betacellulin could possibly initiate or potentiate neovascularization in the diabetic retina.

This hypothesis could explain two well-known, but puzzling clinical observations: (i) Diabetic patients who receive a successful pancreas transplant that improves glucose metabolism and may free them from insulin-dependence rarely show improvement in their retinopathy or in their peripheral vascular disease. We speculate that the patient's original pancreas continues to secrete betacellulin. (ii) Patients who undergo total pancreatectomy for cancer develop severe diabetes because of complete absence of insulin, but they rarely if ever develop diabetic retinopathy, even when they survive for more than 10-20 years. Thus, it is possible that excessive release of betacellulin may contribute to the vascular complications of diabetes. Recent experiments by Bela Anand-Apte of the Cole Eye Institute, Cleveland Clinic, in collaboration with my laboratory, show that in mice with diabetes induced by streptozotocin, intravitreal injection of betacellulin significantly increases vascular leakage in the retina (unpublished data). Betacellulin may provide a biochemical link between pancreatic islets and the microvasculature of the eye. It can be speculated that blockade of betacellulin, perhaps by an antibody, could ameliorate diabetic retinopathy and synergize anti-VEGF therapy.

SUMMARY

Experimental models of ocular neovascularization in the early 1970's made it possible to prove that tumors secreted specific pro-angiogenic proteins. These models also evolved into bioassays to identify novel angiogenesis inhibitors, both endogenous and synthetic. These angiogenesis inhibitors paved the way for the development of a new class of FDA-approved drugs

that have become a "fourth modality" for anti-cancer therapy. These same new drugs have more recently become a novel approach for the treatment of neovascular age-related macular degeneration. Current experiments in many laboratories indicate that in the future, other diseases of ocular neovascularization and/or vascular hyperpermeability may be treated by these angiogenesis inhibitors. Long-term maintenance of suppression of pathological ocular neovascularization may become possible. Angiogenesis-based biomarkers in the blood or urine may be employed to predict patients who are at risk for recurrence of ocular neovascularization, so that treatment can begin before detection by conventional methods. Finally, additional mediators of ocular neovascularization may exist, such as in diabetic retinopathy, where betacellulin is a candidate for study.

Judah Folkman, MD

REFERENCES

1. E. M. Stone, A very effective treatment for neovascular macular degeneration. *N. Engl. J. Med*. **355**, 1493-1495 (2006).
2. J. Folkman, Angiogenesis: an organizing principle for drug discovery? *Nat. Rev. Drug Discov.* **6**, 273-286 (2007).
3. N. Almog, V. Henke, L. Flores, L. Hlatky, A. L. Kung, R. D. Wright, R. Berger, L. Hutchinson, E. Bender, E. Achilles, and J. Folkman, Prolonged dormancy of human liposarcoma is associated with impaired tumor angiogenesis, *FASEB J.* **20**, 947-949 (2006).
4. G. N. Naumov, E. Bender, D. Zurakowski, S. Kang, D. Sampson, E. Flynn, R. S. Watnick, O. Straume, L. A. Akslen, J. Folkman, and N. Almog, A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype, *J. Natl. Cancer Inst.* **98**, 316-325 (2006).
5. G. H. Algire, H. W. Chalkley, F. Y. Legallais, and H. Park, Vascular reactions of normal and malignant tumors in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants, *J. Natl. Cancer Inst.* **6**, 73-85 (1945).
6. I. C. Michaelson, Vascular morphogenesis in the retina of the cat. *J. Anat.* **82**, 167-174 (1948).
7. H. S. N. Greene, Heterologous transplantation of mammalian tissues, *J. Exper. Med.* **73**, 461, (1961).
8. M. Greenblatt and P. Shubik, Tumor angiogenesis: transfilter diffusion. Studies in the hamster by transparent chamber technique, *J. Natl. Cancer Inst.* **41**, 1111, (1968).
9. R. L. Erhman and M. Knoth, Choriocarcioma transfilter stimulation of vasoproliferation in the hamster cheek pouch–studied by light and electron microscopy, *J. Natl. Cancer Inst.* **41**, 329, (1968).

10. J. Folkman, Tumor angiogenesis: therapeutic implications, *N. Engl. J. Med.* **285**, 1182-1186 (1971).
11. J. Folkman, E. Merler, C. Abernathy, and G. Williams, Isolation of a tumor factor responsible for angiogenesis, *J. Exp. Med.* **133**, 275-288 (1971).
12. M. A. Gimbrone, Jr., R. S. Cotran, S. B. Leapman, and J. Folkman, Tumor growth and neovascularization: an experimental model using rabbit cornea. *J. Natl. Canc Inst.* **52**, 413-427 (1974).
13. R. Langer and J. Folkman, Polymers for the sustained release of proteins and other macromolecules, *Nature* **263**, 797-800 (1976).
14. D. H. Ausprunk, K. Falterman, and J. Folkman, The sequence of events in the regression of corneal capillaries, *Lab. Invest.* **38**, 284-294 (1978).
15. Y. Shing, G. Christofori, D. Hanahan, Y. Ono, R. Sasada, K. Igarashi, and J. Folkman, Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor, *Science* **223**, 1296-1298 (1984).
16. Y. Shing, J. Folkman, C. Haudenschild, D. Lund, R. Crum, amd M. Klagsbrun, Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor, *J. Cell. Biochem.* **29**, 275-287 (1985).
17. F. Esch, A. Baird, N. Ling, N. Ueno, F. Hill, L. Denoroy, R. Klepper, D. Gospodarowicz, P. Bohlen, and R. Guillemin, Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF, *Proc. Natl. Acad. Sci. USA* **82**, 6507-6511 (1985).
18. D. Gospodarowicz, Purification of bovine fibroblast growth factor from pituitary, *J. Biol. Chem*. **250,** 2515-2520 (1975).
19. M. Klagsbrun, J. Sasse, R. Sullivan, and J. A. Smith, Human tumor cells synthesize an endothelial cell growth factor that is structurally related to bFGF, *Proc. Natl. Acad. Sci. USA* **83,** 2448-2452 (1986).
20. D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak, Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid, *Science* **219**, 983-985 (1983).
21. N. Ferrara and W. J. Henzel, Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells, *Biochem. Biophys. Res. Commun.* **161**, 851-858 (1989).
22. R. A. Rosenthal, J. F. Megyesi, W. J. Henzel, N. Ferrara, and J. Folkman, Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor, *Growth Factors* **4**, 53-59 (1990).
23. A. P. Adamis, D. T. Shima, K.-T. Yeo, T.-K. Yeo, L. F. Brown, B. Berse, P. A. D'Amore, and J. Folkman, Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells, *Biochem. Biophys. Res. Commun.* **193**, 631-638 (1993).
24. J. W. Miller, A. P. Adamis, D. T. Shima, P. A. D'Amore, R. S. Moulton, M. S. O'Reilly, J. Folkman, H. F. Dvorak, L. F. Brown, B. Berse, T.-K. Yeo, and K.-T. Yeo, Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model, *Am. J. Pathol.* **145**, 574-584 (1994).
25. A. P. Adamis, J. W. Miller, M.-T. Bernal, D. J. D'Amico, J. Folkman, T.-K. Yeo, and K.-T. Yeo, Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy, *Am. J. Ophthalmol.* **118**, 445-450 (1994).
26. L. P. Aiello, R. L. Avery, P. G. Arrigg, B. A. Keyt, H. D. Jampel, S. T. Shah, L. R. Pasquale, H. Thieme, M. A. Iwamoto, J. E. Park, *et al*. Vascular endothelial growth

factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders, *N. Engl. J. Med.* **331**, 1480-1487 (1994).
27. D. T. Shima, A. P. Adamis, N. Ferrara, K.-T. Yeo, T.-K. Yeo, R. Allende, J. Folkman, and P. A. D'Amore, Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen, *Mol. Med.* **1**, 182-193 (1995).
28. A. P. Adamis, D. T. Shima, M. J. Tolentino, E. A. Gragoudas, N. Ferrara, J. Folkman, P. A. D'Amore, and J. W. Miller, Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate, *Arch. Ophthalmol.* **114**, 66-71 (1996).
29. J. S. Penn, V. S. Rajaratnam, R. J. Collier, and A. F. Clark, The effect of an angiostatic steroid on neovascularization in a rat model of retinopathy of prematurity, *Invest. Ophthalmol. Vis. Sci.* **42**, 283-290 (2001).
30. E. M. Stone, A very effective treatment for neovascular macular degeneration, *N. Engl. J. Med.* **355**, 1493-1495 (2006).
31. A. G. Marneros, H. She, H. Zambarakji, H. Hashizume, E. J. Connolly, I. Kim, E. S. Gragoudas, J. W. Miller, and B. R. Olsen, Endogenous endostatin inhibits choroidal neovascularization, *FASEB J.* **21**: (published online, May 25, 2007).
32. K. S. Moulton, E. Heller, M. A. Konerding, E. Flynn, W. Palinski, and J. Folkman, Angiogenesis inhibitors endostatin and TNP-470 reduce intimal neovascularization and plaque growth in Apolipoprotein E-deficient mice, *Circulation* **99**, 1726-1732 (1999).
33. K. S. Moulton, B. R. Olsen, S. Soon, N. Fukai, D. Zurakowski, and X. Zeng, Loss of collagen XVIII enhances neovascularization and vascular permeability in atherosclerosis, *Circulation* **110**, 1330-1336 (2004).
34. A. Abdollahi, P. Hahnfeldt, C. Maercker, H. J. Gröne, J. Debus, W. Ansorge, J. Folkman, L. Hlatky, and P. E. Huber, Endostatin's antiangiogenic signaling network, *Mol. Cell* **13**, 649-663, (2004).
35. A. Abdollahi, C. Schwager, J. Kleeff, I. Esposito, S. Domhan, P. Peschke, K. Hauser, P. Hahnfeldt, L. Hlatky, J. Debus, J. M. Peters, H. Friess, J. Folkman, and P. E. Huber, A Transcriptional Network Governing the Angiogenic Switch- Evidence in Human Pancreatic Carcinoma, *Proc. Natl. Acad. Sci. USA* 2007, in press.
36. R. Roy, U. M. Wewer, D. Zurakowski, S. E. Pories, and M. A. Moses, ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage, *J. Biol. Chem.* **279**, 51323-51330 (2004).
37. G. Klement, L. Kikuchi, M. Kieran, N. Almog, T. T. Yip, and J. Folkman, Early tumor detection using platelet uptake of angiogenesis regulators. Proc 47th American Society of Hematology. *Blood* **104**, 239a Abs. #839 (2004).
38. G. Klement, D. Cervi, T. Yip, J. Folkman, and J. Italiano, Platelet PF-4 Is an early marker of tumor angiogenesis, *Blood* **108**, 426a, Abs. #147 (2006).
39. Y. Shing, G. Christofori, D. Hanahan, Y. Ono, R. Sasada, K. Igarashi, and J. Folkman, Betacellulin: A novel mitogen from pancreatic beta tumor cells, *Science* **259**, 1604-1607 (1993).
40. Y. Shing and J. Folkman, Betacellulin, in: *Human Cytokines, Handbook for Basic and Clinical Research, Vol II*, edited by B. B. Aggarwal and J. U. Gutterman (Blackwell Scientific Publications, Inc., 1996) pp. 331-339.

ANGIOGENESIS STUDY MODELS

Chapter 1

CELLULAR AND MOLECULAR MECHANISMS OF RETINAL ANGIOGENESIS

What have we learned from in vitro models?

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Abstract: Angiogenesis is a multi-factorial process that involves different cell types and a number of cytokines and growth factors. Physiological angiogenesis is characterized by the existence of a delicate balance between pro-angiogenic and anti-angiogenic factors. In an *in vivo* setting, pro-angiogenic stimuli such as endothelial-specific mitogenic factors and extracellular matrix (ECM)-degrading enzymes must be tightly regulated and locally constrained. Differentiation factors, protease inhibitors, and the elements involved in reconstruction of ECM and recruitment of mural cells must be elicited in an appropriate temporal and spatial arrangement. Overexpression of angiogenesis-activating factors may cause hyper-vascularization. However, deficiency or disarray in expression of anti-angiogenic factors may result in leaky vessels, unstable capillaries, and formation of dysfunctional neovascular tufts as seen in retinopathy of prematurity, diabetic retinopathy, or other conditions of retinal neovascularization. In other words, pathological angiogenesis is characterized not only by excesses in pro-angiogenic factors but also an insufficiency in anti-angiogenic, pro-differentiation factors.

To better understand pathological angiogenesis, our experimental models should be able to dissect the *dissolution* phase of the angiogenic process from the *resolution* phase. In an *in vivo* model of pathological angiogenesis, these two components occur in close spatial and temporal proximity and thus are difficult to dissect. By using *in vitro* models, it is possible to begin with the most basic elements in order to reconstitute physiological and pathological conditions and compare each step of the process.

Retina explants, primary cultures of retinal vascular endothelial cells, and co-cultures of endothelial and mural cells, together with gene transfer techniques, have enabled us to analyze the functional roles of cytokines, growth factors,

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and extracellular proteolytic enzymes involved in the angiogenic process and to develop assay systems for testing the efficacy of pharmaceutical reagents that specifically block intracellular signaling pathways and transcription factors. Finally, use of endothelial and mural cells isolated from transgenic animals in tissue culture models aids in defining gene functions and elucidating the mechanisms of their regulation.

1. INTRODUCTION

Much of our knowledge about retinal neovascularization has been obtained from research done with animal models. In addition to retinopathies in naturally occurring rodent mutants, a number of pathologies representing human disease conditions, such as retinopathy of prematurity, age related macular degeneration, and diabetic retinopathy, can be modeled in normal and transgenic animal retinas to study morphological aspects of abnormal angiogenesis. Analysis of diseased retinas using advanced imaging techniques, confocal microscopy, immunohistochemistry, *in situ* hybridization, and laser-capture micro-dissection has uncovered cellular elements and biological factors involved in the initiation and progression of pathological angiogenesis. However, the precise cellular sources and targets of gene and protein expression, as well as their molecular regulation and mechanisms of action, are more readily identified using *in vitro* models. Anti-angiogenic or pro-angiogenic reagents are best characterized for target specificity, effective dose and treatment time, potential cell toxicity, and mechanism of action when tested on isolated cells or organ explants under well defined tissue culture conditions.

Retinal neovascularization, the inappropriate proliferation of new vessels derived from preexisting vessels, is a major cause of blindness and a significant component of many ocular diseases. Different types of cells participate in retinal angiogenesis, including endothelial cells, pericytes, astrocytes and Muller glial cells (see Figure 1). Hypoxia and hyperglycemia have been shown to be important causes of retinal neovascularization. In both conditions, the balance between angiogenic and angiostatic growth factors, which usually serves to keep angiogenesis in check, is disturbed.

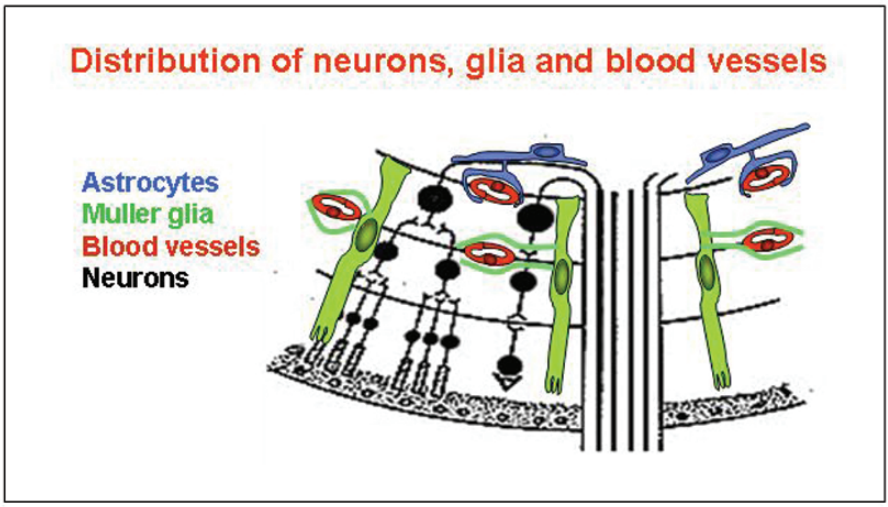

Figure 1-1. Distribution of neurons, glia (Muller cells and astrocytes), and blood vessels within the retina. Color coding indicates the type of cell.

1.1 Angiogenic Process

The purpose of this introduction is to depict the multi-factorial/multi-cellular nature of the angiogenic process and show how *in vitro* models can be used to dissect and identify the individual elements involved and analyze their mechanism of action. Angiogenesis is defined as the formation of new blood vessels by sprouting from pre-existing capillaries. Physiological angiogenesis takes place during development and wound healing and in the female reproductive system. Pathological angiogenesis is manifested in proliferative retinopathy, hemangioma, psoriasis, and atherosclerosis and in the growth and metastasis of solid tumors.

Angiogenesis is an intricate process that is regulated by many growth factors and cytokines. Angiogenesis-regulating factors can be classified into two groups based on whether they are involved in the activation or *dissolution* phase, which begins in the endothelium of pre-existing vessels, or the *resolution* phase, which results in differentiation of the newly formed capillaries. The *dissolution* phase of angiogenesis begins with expression or induction of proteolytic activities in otherwise quiescent endothelial cells, leading to breakdown of the cell junctions, increases in paracellular

permeability, and degradation of the underlying basement membrane. The activated endothelial cells are thus set free to penetrate the surrounding tissue as they migrate and proliferate along a concentration gradient towards the source of angiogenic stimuli. Ischemia and hypoxia have been identified as major stimuli for angiogenesis in developing embryos, developing retinas, and growing tumors. A variety of angiogenic factors and cytokines including vascular endothelial growth factor (VEGF), angiopoietin-1, basic fibroblast growth factor (b-FGF), tumor necrosis factor alpha (TNF-alpha), transforming growth factor-beta (TGF-beta), and platelet derived growth factor (PDGF) have been identified in these tissues (for review, see[1-4]). In addition to the initial stimuli, which originate at distal sites, pro-angiogenic elements appear rapidly within the *dissolution* milieu. These include growth factors, pro-enzymes, and other serum proteins that leak from destabilized capillaries; extracellular matrix (ECM)-bound growth factors that are released or activated by proteases; and endothelial precursor cells that are recruited from the bone marrow or circulation.

Depending on the tissue in which angiogenesis is taking place and whether the angiogenesis is physiological or pathological, other cellular elements in addition to endothelial cells may participate in the process. In the developing retina for example, astrocytes invade the retina from the optic nerve to guide the migrating endothelial cells.[5-7] As endothelial cells elongate and assemble into a meshwork of interlaced cords, the *resolution* phase of angiogenesis begins with cessation of endothelial cell proliferation, induction of endothelial cell differentiation/lumen formation, and reconstruction of the vascular basement membrane. Redundant connections and excess branches are pruned, probably by a precisely localized apoptosis that involves leukocytes.[8] The meshwork-like interconnecting tubes and tufts are reduced to a pattern of branching, bifurcated vessels with efficient directional blood flow. Critical to the stabilization of the newly formed capillaries is the recruitment of pericytes, which wrap around the vessels.[9]

A variety of angiogenic inhibitors have been implicated in the *resolution* phase of angiogenesis, including endostatin, angiostatin, thrombospondin, interleukin-1, interferon-beta, prostate-specific antigen, tissue inhibitors of metalloproteinases (TIMP), angiopoietin-2, pigment epithelial derived factor (PEDF), and TGF-beta. The role of TGF-beta in the *resolution* phase of angiogenesis has been studied extensively. *In vitro*, TGF-beta has been shown to inhibit endothelial cell proliferation and migration,[10] whereas it promotes formation of capillary-like tubules in three-dimensional matrix gels.[11,12] Studies by several groups have demonstrated that TGF-beta has a biphasic effect on endothelial cells. Depending on the concentration, it either inhibits or potentiates endothelial cell proliferation, invasion, or tube formation in three-dimensional collagen gels.[13-15] Similarly, the dose-dependent,

synergistic and/or antagonizing effects of angiopoietin (types 1 and 2) and VEGF have been the focus of numerous investigations.[16-19]

The seemingly paradoxical effects of the angiopoietins or TGF-beta observed in cultured endothelial cells are good testimony to the importance of *in vitro* approaches for understanding the mechanisms of cytokine actions in the angiogenesis process *in vivo*. For example, the biphasic function of TGF-beta *in vitro* suggests that it plays a dual role *in vivo* as well, acting as an activating factor to promote angiogenesis at the lower end of its concentration gradient near the sprouting vascular stalk and as a resolution factor to inhibit proliferation and promote differentiation at the upper end of its concentration spectrum near the site of ischemia/hypoxia. Conversely, a cytokine may act as an angiogenic activator at the sprouting site where it is released at high concentrations, but it may function as a 'resolution' factor at locations distal to the angiogenic sprout where it is present at lower concentrations.

It is important to recognize that, in an *in vivo* setting, the *dissolution/ activation* phase of angiogenesis is closely followed by the *resolution/ differentiation* phase. This means that mitogenic and migratory factors, together with ECM-degrading enzymes, must be tightly regulated and locally constrained. The angiostatic, pro-differentiation factors, protease inhibitors, and other elements involved in reconstruction of ECM and recruitment of capillary-stabilizing mural cells should be able to function in close proximity to the activating 'destabilizing' factors. Much as the over-expression of activating factors may cause hyper-vascularization, deficiency (or mal-distribution) of *resolution* factors may be associated with leaky unstable capillaries and formation of dysfunctional vascular tufts in pathological situations. In other words, pathological angiogenesis can be characterized not only by excessive pro-angiogenic factors but also by an insufficient supply of anti-angiogenic, pro-differentiation factors.[2-4]

To better understand angiogenesis, our experimental models should enable us to dissect and separately manipulate the *dissolution* and *resolution* phases of this intricate process. In pathological angiogenesis, these two components occur in close spatial and temporal proximity, and, thus, are difficult to monitor. In order to model pathological angiogenesis, we should begin with the most basic elements and reconstitute the physiological and pathological conditions side-by-side and step-by-step. Endothelial cells are the essential element of angiogenesis. They form the lumen of blood vessels and function in a number of local roles, including control of vascular tone, provision of an anticoagulant surface, maintenance of the blood/tissue barrier and defense against inflammatory cells. Preparation of vascular

endothelial cells in culture is the first step in developing the models necessary to characterize physiological and pathological angiogenesis.

1.2 Primary Cultures of Endothelial Cells

Jaffe and co-workers[20] were probably the first to isolate endothelial cells on a large scale. They prepared and characterized human umbilical vein endothelial cell (HUVEC) cultures using a combination of morphologic, electron-microscopic, and functional assays. The cells were grown as a homogeneous population for up to 5 months or were subcultured for 10 serial passages, although cell growth rate was rather slow (doubling time of 92 hours). Using the same methods, Gimbrone's group[21] prepared HUVEC cultures for studies of endothelial growth and migration and suggested the potential value of these cells as an *in vitro* model of angiogenesis. Large vessel endothelial cells from aorta or pulmonary arteries have been prepared in many laboratories using similar methods.[22,23] The vessel can be filled with a solution of collagenase and incubated until the endothelial cells are released; alternatively, large vessels are cut open so that the interior endothelial layer can be removed by gentle scraping.

Endothelial cells isolated from different areas of the vascular tree have diverse characteristics. Studies indicate that this diversity is due, in part, to micro-environmental influences. In culture, endothelial cells are capable of acquiring new properties depending on the characteristics of the plating surface and the culture medium.[24,25] Milici *et al*. reported that bovine adrenal capillary endothelial cells cultured on plastic exhibited very low levels of diaphragmed fenestrations and almost no transendothelial channels as compared to cells grown on basal lamina.[26] The ability of CNS endothelial cells to form a blood-brain barrier is thought to depend in large part on factors present in the CNS environment.[27,28] That is, endothelial cells can be manipulated in culture to develop a specific phenotype and to satisfy the requirements of a particular functional assay. Despite this apparent plasticity of vascular endothelial cells, use of capillary endothelial cells is preferred for developing models of angiogenesis because the capillary vessel, not the large vessel, is the origin of new sprouts.

Isolation of endothelial cells from capillaries has proven to be more difficult than isolation from large vessels. A large-scale preparation of capillary endothelial cells and pericytes from bovine cerebral cortex was reported first by Carson and colleagues in 1986.[29] Microvascular endothelial cells were also isolated from rat epididymal fat pad and characterized for their growth on different substratum by Madri and Williams.[30] Kern and colleagues isolated microvascular endothelial cells from human adipose tissue,[31] and Marks and co-workers described an improved method of

isolating human dermal microvascular endothelial cells from foreskin using percoll density gradient centrifugation. These authors examined the use of serum and other growth factor supplements for improving culture conditions.[32] Mouse brain endothelial cells have been prepared by several groups.[33,34]

There are a number of good protocols for preparation of endothelial cells from retina. Buzney and Massicotte were probably the first to report that capillaries isolated from fetal calf retina give rise to endothelial cell colonies in culture.[35] Frank and co-workers reported on growth of microvascular endothelial cells from kitten retina.[36] Large-scale preparation of primary cultures of microvascular endothelial cells from bovine retina was reported by Bowman and co-workers[37] and by Capetandes and Gerritsen.[38] The latter group explained the advantage of using fibronectin coated dishes and plasma-derived serum supplement. The plasma-derived serum preparation, also designated as platelet-poor plasma, is a preferred medium supplement for endothelial cells because it does not support the growth of contaminating pericytes. Retinal endothelial cells have also been isolated from rhesus monkey,[39] human,[40] and mouse.[41]

Our laboratory has been using bovine retinal capillary endothelial (BRE) cells for several years.[42-46] To isolate BRE cells, we follow the method of Bowman[37] as improved by Capetandes[38] and Laterra.[47] The retinal tissue is homogenized, and small capillaries are collected over a nylon sieve (80 μm). The material retained by the sieve is briefly digested by collagenase and plated in dishes precoated with collagen and fibronectin. Using platelet-free serum favors endothelial cell growth over contaminating cells. However, if contaminating cells are overwhelming, individual endothelial colonies are isolated and pooled into a new sub-culture. In the initial culture, some colonies grow faster than the others. As a result, the subcultures may not represent the actual heterogeneity of the *in vivo* cell population. Nevertheless, depending on the purpose of experiment, one may compromise by using less homogenous cultures of early passage, or pure endothelial cells of later passage. Other parameters, such as cell density and the proliferative versus quiescent state of the cells, should be considered in such assays as cell proliferation, cell migration, or programmed cell death.

1.3 Naturally Transformed and Conditionally Immortalized Endothelial Cell Lines

Retinal endothelial cell cultures are most commonly prepared by isolation and enzymatic disruption of microvessels. The preparation is then cultured, and individual colonies of endothelial cells are isolated from the more

aggressively growing pericytes and pooled into a pure endothelial subculture. Alternatively, the digested preparation is further enriched for endothelial cells by gradient centrifugation or selection by the use of endothelial cell surface-specific markers. Laboratory mice are a good source of retinal endothelial cells. However, isolation and maintenance of a primary culture of retinal or cerebral endothelial cells from small rodents is difficult due to the limited tissue supply. On the other hand, the wealth of information about the genetics and developmental biology of laboratory mice and the availability of transgenic mouse models provide a strong motivation for improving methods of preparing durable cultures of microvascular endothelial cells from the mouse. Genetically modified mouse strains can now be used to study the functional role of a particular gene (or gene mutation) in the manifestation of vascular disease in retina. Once such a causal relationship is established *in vivo*, cultured vascular cells from the transgenic mouse offer an excellent opportunity to analyze mechanisms of the gene's regulation and function.

Several laboratories have isolated clones of spontaneously transformed endothelial cells with a wide range of characteristics from mouse or rat retinas. Others have transformed primary endothelial cultures by introducing exogenous oncogenes. The advantage of the immortalized cell lines is that there is no need for a periodic preparation of primary cultures. The disadvantage is that some morphological or functional characteristics, such as reduced requirement for serum supplement and growth factors, lack of response to cytokines, or compromised barrier function, may render them unsuitable for a particular experimental protocol. For example, DeBault and co-workers cloned an endothelial cell line from mouse brain, designated ME-2, that retained many properties of primary cultures, including growth characteristics and specific cell surface antigens for up to 40 passages before becoming senescent.[48] On the other hand, Robinson and co-workers isolated a spontaneously transformed clone of mouse brain endothelial cells, designated Ten, that exhibited characteristics of transformed cells, including growth in serum-free medium, anchorage-independent growth, tumorigenicity in nude mice, and lack of contact inhibition. Nevertheless, Ten cells maintained endothelial cell markers and responded to EGF and PDGF mitogenic activities.[49]

Comparing the preparations of DeBault and Robinson, it appears that there is neither a simple formula for preparation nor a standard inclusive definition of a cloned endothelial cell line that is qualified to serve as an experimental model for all kinds of assays. A cell line with reduced serum requirements is not the best choice for testing mitogenic factors, and a clone lacking contact inhibition would not serve well for studies of cell migration or permeability. One important criterion for the general suitability of cloned

endothelial cultures would be their potential to senesce after a number of passages. Any cell preparation, whether it originated from a single colony or from several pooled colonies, can be considered as closer to primary when, over a number of passages, the growth rate declines and some large, multi-nucleated (fried-egg like) non-proliferating cells appear in the culture. Of course, mishandling cultures can also give rise to giant non-proliferating cells. Retinal endothelial cells fed on a regular basis and transferred at high density (at a ratio of 1:3 surface areas) can be maintained for up to 12-14 passages before senescent cells begin to appear in the culture. Moreover, depending on how successful the initial plating is, the culture may better represent the primary population of cells if only the contaminating cells are removed. On the other hand, naturally transformed endothelial cell colonies may be isolated and pooled from passages of the primary culture. These cells, while not representing the primary population of cells, may be used for a particular experiment if they are carefully characterized.

Finally, one may modify the culture condition to optimize the use of transformed cell lines for a particular assay. The paracellular leakiness of transformed endothelial cells, which can be readily tested by measuring the transendothelial electrical resistance (TER), has been suppressed by the use of astrocytes as co-culture or astrocyte-conditioned medium as supplement.[50,51]

A number of brain capillary endothelial cell lines have been isolated from mouse or rat carrying the temperature sensitive version of the Simian Virus-40 large tumor antigen (SV-40 Tag). SV-40 Tag is an oncogene that causes cells to grow continuously in the absence of growth factors. At the permissive temperature (33 °C), the cells show transformed characteristics and replicate continuously. When switched to 37-39 °C, the expression of SV-40 Tag is halted, and the cells exhibit characteristics of primary cultures. Using magnetic beads coated with anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody, Su and co-workers isolated endothelial cells from wild-type and thrombospondin-1 deficient ($TSP1^{-/-}$) mice carrying the SV-40 Tag gene (transgenic immorto-mouse).[41] Both cell lines expressed endothelial cell markers, but $TSP1^{-/-}$ cells were deficient in their ability to form capillary-like networks on Matrigel, confirming the known anti-angiogenic role of thrombospondin. This method would be extremely useful for comparing the physiological role of specific genes in endothelial cell function.

Magnetic beads coated with PECAM-1 antibody have been used for isolating retinal endothelial cells from Lewis rats,[52] and retinal pericyte cell lines expressing the SV-40 Tag have also been isolated from the SV-40 Tag rat strain.[53] The cells grew exponentially at the permissive temperature of

33 ºC, but became quiescent within 2 days when shifted to 37 ºC. Several immortalized brain capillary endothelial cell lines also have been established from transgenic mice harboring the SV-40 Tag.[54,55]

Reversion of immortalization seems to be necessary in some experimental models where the immortalizing gene may influence the experimental outcome.[56] In H-2Kb-tsA58 transgenic mice, the temperature sensitive SV-40 Tag gene is controlled by the H-2k(b) class-I histocompatibility promoter, which is inducible by INF-gamma. Using this mouse, Lindington and co-workers established a cardiac endothelial cell line, which grew exponentially at the permissive temperature and in the presence of INF-gamma.[57] At 38 ºC and in the absence of INF-gamma, the cells stopped growing and became responsive to basic FGF, VEGF, and EGF. By cross-breeding this same mouse with the uPAR$^{-/-}$ mouse, we have been able to isolate uPAR$^{-/-}$ brain endothelial cell lines; characterization of these cells is underway in our laboratory (Behzadian *et al*., unpublished). Urokinase plasminogen activator and its receptor (uPA/uPAR) have been implicated in the regulation of endothelial barrier function and endothelial cell migration. Isolation of mutant endothelial cells from transgenic uPAR$^{-/-}$ mice provides an important *in vitro* model for studying the function of the uPA/uPAR system in retinopathy.

1.4 Endothelial Precursor Cells

The process of angiogenesis, defined as formation of new blood vessels by sprouting from pre-existing blood vessels, has been classically distinguished from vasculogenesis, which is the mobilization and assembly of mesenchimal endothelial precursor cells into vascular structures. This distinction was based not only on differences in the way the two processes occur, but also on the notion that vessel growth during embryonic development mainly involves vasculogenesis, whereas postnatal neovascularization occurs mainly by angiogenesis. However, recent evidence indicates that vasculogenesis plays a significant role in postnatal neovascularization.[58-60] The identification of endothelial precursor cells in the adult bone marrow and circulating blood[61] led to the discovery of endothelial precursor cells in sites of neovascularization and changed our understanding of postnatal vessel growth and repair.[62] Active recruitment of endothelial precursor cells has been demonstrated in the ischemic retina,[63-67] suggesting that therapies targeting these precursor cells will help in blocking pathological neovascularization in retina.

Studies of bone marrow-derived hematopoietic stem cells (HSCs) and endothelial progenitor cells are thoroughly discussed elsewhere in this volume. Our focus is on potential use of endothelial precursor cells for *in*