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Angiogenesis-Based Dermatology

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ISBN 978-1-4471-7312-0 ISBN 978-1-4471-7314-4 (eBook)
DOI 10.1007/978-1-4471-7314-4

Library of Congress Control Number: 2017933481

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer-Verlag London Ltd.
The registered company address is: 236 Gray's Inn Road, London WC1X 8HB, United Kingdom

Preface

This book is dedicated to the memory of Judah Folkman, MD, with whom I had the pleasure of spending a 4-year postdoctoral fellowship, between the years 1994 and 1998. There are several aspects to Dr. Folkman's personality that made him so effective in advancing angiogenesis in medicine. First, his research was clinically driven. He wanted to use angiogenesis as a tool to cure human disease. Thus, he chose to use basic research to address clinical problems, not as an end to itself. There are two camps of people who perform basic research with regard to human disease. The first believes that we have to know everything in order to treat human disease. A corollary of that belief is that once we know everything, we will be able to design a specific targeted therapy that cures advanced cancer or other ailment, with no side effects. This will be accomplished because advanced cancers are addicted to an oncogene, and targeting that oncogene will lead to a painless cure. The second camp is the one that Dr. Folkman belonged to, in that he wanted to know how we could leverage the knowledge that we have today to help patients who are sick today. As a pediatric surgeon, he recognized that patients who are sick today need treatments today, and will likely not survive until that utopian time that we have magic bullets with no side effects. His concepts of angiogenesis inhibition, first expounded in *The New England Journal of Medicine* in 1971, were fiercely attacked both at home and in other institutions. He had the courage to take these to clinical trials, which were also controversial, but led to new treatments of cancer and disorders of excess vasculature. He was always in a hurry because he knew that time was short and that people are sick and don't have infinite lifespans. He was also very open minded and allowed anyone to pursue their interests as long as they were related to angiogenesis. In this vein, he provided critical advice to a generation of scientists in terms of career advancement. Finally, he was a total gentleman, and never disparaged those who attacked and ridiculed him, although we knew very well the identities of these individuals. The term "noble metal" refers to metals that are resistant to corrosion, like gold. Dr. Folkman was resistant to the corrosive environment that he lived in his entire professional life. This makes him noble.

Lab meetings in the Folkman lab were Friday mornings. I personally felt as if we were knights at the Round Table of Angiogenesis, each of us representing a separate field of angiogenesis. Studies from those heady times led to the development of thalidomide for myeloma, anti-VEGF therapy for macular degeneration, understanding of the relationship of oncogenic signaling, understanding of the biology of

hemangiomas and vascular malformations, isolation of endogenous angiogenesis inhibitors, urinary detection of angiogenic biomarkers, angiogenesis and metabolism, and many other advances that are in the clinic today. Dr. Folkman had a way of quieting skeptics. When someone said, that won't work, he gave him a pen and piece of paper and told him to write it down and commit to it. I never saw anyone actually committing their skepticism to paper.

Many of us alumni in the Folkman lab wonder how he would view the world today. As someone who knew him from 1994 to the time of his untimely death in 2008 in the Denver airport, I can venture some predictions. He would be thrilled with the clinical discovery that propranolol, a very old drug, is highly effective against hemangiomas of infancy, and has been applied to this condition, even though the mechanism of propranolol against infantile hemangiomas is not fully understood. He would also be thrilled by the use of rapamycin against vascular malformations. Part of the rationale for using rapamycin on vascular malformations was our discovery that mTOR is activated in vascular malformations, which we demonstrated on paraffin samples which I had acquired while in his laboratory.

Dr. Folkman would not be surprised by the failure of the oncogene addiction hypothesis, because as a surgeon, he had seen numerous advanced tumors and intuitively knew that large solid tumors were not reliant on a single oncogene. Evidence of this are the numerous mutations and mechanisms that have been reported in Braf inhibitor resistance in advanced melanoma. He would be pleased with the use of Avastin as a treatment for macular degeneration and cancer, although he would recognize that our use of Avastin causes compensatory events in solid tumors that require further therapies, such as those that target NADPH oxidases and HIF2a, a response to chronic hypoxia. He would likely be disappointed in the failures of angiostatin and endostatin in the clinic, and would call for further studies to understand why these drugs didn't show the same effect in humans as they did in mice. He would be gratified about the role of angiogenesis inhibition in promoting antitumor immunity, and would call for combinations of angiogenesis inhibitors and checkpoint inhibitors against solid tumors. Finally, he would approve of the concept of angioprevention, the use of angiogenesis inhibitors to prevent the formation of cancer, which is widely practiced today by the public with natural products.

Every dermatologist who prescribes a drug has a little bit of Dr. Folkman in them. When we prescribe a drug, we do not know the full mechanism of the action of the drug. Doxycycline kills bacteria but also inhibits matrix metalloproteinases. Which is more important for treatment of acne and rosacea? We don't know, but we don't let our lack of knowledge serve as an excuse not to treat patients. It is my hope that the well-written chapters in this book will serve to clarify to the practicing dermatologist a more full understanding of their every day actions. Once the dermatologist in the trenches has a better understanding of what they do, they too can contribute to the immediate advancement of knowledge through keen clinical observations which can be rapidly disseminated and aid treatment of patients today.

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Angiogenesis: General Concepts

1

L. Jensen and Y. Cao

Introduction

The process of blood vessel growth, generally referred to as "angiogenesis", is pivotal for the development, homeostasis and function of all tissues and organs in the organism, but also for progression of most serious diseases including ophthalmic disorders, cancer, cardiovascular disorders, inflammatory disorders and chronic skin ulcers [1]. In the last decades, great progress has been made in our understanding of the mechanisms that regulate angiogenesis and vascular functions in health and disease, and this continues to be one of the largest areas of research today [2]. It is now clear that the regulation of tissue/organ development, physiology and disease progression by blood vessels is highly complex and context-dependent, but certain general concepts and factors are central and important for angiogenesis during development and in most diseases. Such general concepts will be the focus of this chapter.

The concept of blood vessel formation and growth was first mentioned by the Greek philosopher Aristotle in the fourth century BC [3], but the concept of angiogenesis was not thoroughly studied until in the work of John Hunter who has been credited (perhaps erroneously) with introducing the term "angiogenesis" in 1787 [4]. The process and mechanisms regulating angiogenesis was however first

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J.L. Arbiser (ed.), *Angiogenesis-Based Dermatology*,
DOI 10.1007/978-1-4471-7314-4_1

systematically studied using modern in vitro and in vivo assays in the work of angiogenesis pioneer Judah Folkman [5]. Physiological, or healthy angiogenesis is mostly considered as a part of organ development, blood vessels in adults are considered to be mostly quiescent and primarily serve structural purposes as the cells forming the innermost layer of blood vessels. In pathological conditions, such as in cancer, blood vessel growth may, however, be induced in adults, a finding reported as early as in the fourth century BC by Hippocrates [5] and expanded radically in the work performed in the Folkman Lab [2]. The identification of key angiogenic factors including vascular endothelial growth factor (VEGF) in 1989 [6, 7] and the VEGF receptor (VEGFR) in 1990–92 [8–11] and their regulation, as well as the development of a palette of in vitro and in vivo assays suitable for studying mechanisms regulating angiogenesis [5] led to a more mainstream interest in the field, followed by an explosion in the number of publications related to angiogenesis in various aspects of health and disease. It is now clear that angiogenesis is important for all types of tissue growth including during development but also during regeneration of wounds or damaged myocardial or brain tissues following ischemic insults [12, 13] as well as adipose, muscle or tumor growth [14–16]. Most of the work on elucidating the importance of angiogenesis in health or disease, come from studies where the process has been inhibited and subsequent phenotypes analyzed. For example, mice lacking even just a single allele of the important angiogenic factor VEGF-A die in the womb just a few days after the first blood vessels should have been formed, due to massive vascular insufficiency, improper formation of the few vessels that do develop leading to extensive hemorrhage [17]. As a consequence of these vascular defects, mouse embryos stop developing from day E8.5, when the embryo start to require oxygen transport by its own vasculature to maintain oxygenation of the growing and developing organism, and eventually die at E10.5 [17]. Also during post-natal development VEGF-A is important for physiological growth and morphogenesis of the retinal vessels in mice [18], a process that is dependent on hypoxia-induced VEGF-A in the peripheral retina as in animals such as zebrafish that develop in the absence of tissue hypoxia, the closely related homologue VEGF-B is required for retinal angiogenesis [19]. Since these early discoveries, genetic knock-out studies have been used to identify a host of factors necessary for normal developmental angiogenesis or healthy vascular morphogenesis [20]. In addition, the potential of factors to induce ectopic angiogenesis in the otherwise avascular cornea has generated very important knowledge on factors that could be exploited for pro-angiogenic treatment [21–23] alternatively to identify the complex mechanisms involved in angiogenesis under pathological situations such as in tumors [24, 25]. In cancer, quantitative analyses of gene-expression profiles or protein abundance have identified numerous putative angiogenic factors over-expressed in solid tumors of all origins [2]. In particular VEGF-A and dual pro-angiogenic and inflammatory cytokines such as TNF-alpha [26], TGF-beta [27], various interleukins [28–30], chemokines and bone-marrow growth factors [31] as well as extracellular matrix modulating enzymes [32] have been studied along with the putative intracellular mediators. The importance of these factors have then been demonstrated by loss- or gain-of-function studies using mouse tumor models, in which tumor cells or whole animals have been genetically modified to produced

pathologically relevant levels of these various factors. With so many potentially important pro-angiogenic factors identified to date, a key issue is to understand the interplay and synergistic mechanisms that drive tumor angiogenesis in complex environments where several factors are over-expressed at the same time. As such there are likely synergies between various angiogenic factors, that stem from their actions on the different cell-types needed for angiogenesis including endothelial cells, peri-vascular cells and macrophages which complicate using single specific neutralizing or agonistic agents in anti- or pro-angiogenic therapy. In keeping with this idea, single-agent treatments have largely failed both for anti-cancer therapy and for restorative angiogenesis in ischemic disorders. Combinations of pro- or anti-angiogenic drugs thus have much stronger potential in pre-clinical settings [33], but such combination therapies remains to be thoroughly tested in clinical studies.

In many cases angiogenic factors may act in a tissue-dependent manner. I.e. the factors that is important for growth of blood vessels in the brain or eye, such as Wnt7a/b, Grp124 [34, 35] or VEGF-B and Neuropilin1 [19] seem to have little if any role in angiogenesis in other parts of the organism. Similarly, factors regulating angiogenesis in the adipose or muscle tissue coinciding with the growth of these tissues in adults, may also be different from those regulating angiogenesis in for example the skin during wound healing. Leptin, produced at high amounts by mature adipocytes, is a potent angiogenic factor [36], but also more general pro-angiogenic molecules such as VEGF-A is important for adult adipose angiogenesis and blocking these factors pharmacologically inhibit diet-induced or leptin-deficiency-induced obesity in mice [37, 38].

In this chapter we aim to give an overview of the key features of blood vessels, their structure and function and how blood vessels are involved in regulation of tissue functions. In the second part we will focus particularly on the mechanisms regulating vessel growth, maturation and regression, and discuss key differences between the growth and function of healthy versus pathological vessels. Finally we will introduce the concept of lymphangiogenesis and how lymph vessels develop, function and may contribute to disease under pathological conditions.

Blood Vessel Physiology and Function

The first and foremost function of blood vessels is to supply oxygen and nutrients (mainly glucose and lipids) to support cellular metabolism. A large fraction of the blood is therefore dedicated to transport of oxygen via hemoglobin-containing erythrocytes that are responsible for approximately 50% of the blood volume. In the cell-free fraction, the most abundant proteins including albumin and lipoproteins are dedicated to transport of water in-soluble lipids/amphiphiles, and the plasma has a high buffer capacity as a way to combat acidosis or reactive oxygen species that are unavoidable side-effects from oxidative cellular metabolism. The abundance of these oxygen- or lipid-binding factors in the blood also reflect the facts that free oxygen is among the most reactive compounds in the organism and because free lipid is detrimental to vascular physiology. Blood is delivered through the

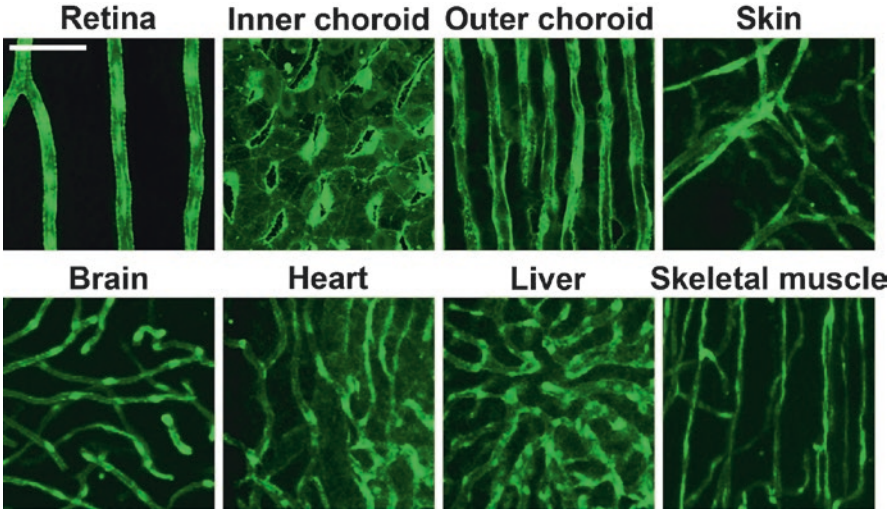

Fig. 1.1 Morphological differences between capillary beds of various tissues. Confocal micrographs of blood vessels, shown in *green*, found in the retina, inner and outer choroid, skin, brain, heart, liver and skeletal muscle of adult transgenic Tg(fli:EGFP)^{y1} zebrafish, expressing enhanced green fluorescent protein (EGFP) in endothelial cells. The white size bar indicate 100 μm

vasculature to all metabolically active tissues and collected by veins which bring waste products, primarily carbon dioxide (CO_2), urea and other metabolites through the kidneys and the liver to the lungs, where the last of the waste products (CO_2) is released and the blood is reoxygenated. This constant circulation of blood is necessary for its function – blood that accumulate in tissues due to insufficient venous drainage, persistent circulation in futile loops or hemorrhage rapidly loose the tissue-supportive function and rather become a reservoir for tissue-damaging factors including cytotoxic cells and compounds [39]. As such, blood is only beneficial to tissues if it is also effectively collected and recycled.

The metabolic activity of a tissue is the primary regulator of blood perfusion. When metabolic activity increase this will lead to tissue hypoxia, which is the most important physiological signal for increasing perfusion. This is perhaps most clearly exemplified in the brain where the activity of different brain regions is directly coupled to changes in perfusion; all techniques used to measure brain activity today actually measure blood perfusion and not the activity of the nerves or other brain cells themselves [40]. In tissues which exhibit constantly high metabolic activity including the liver and the retina, blood vessel density is high compared to other tissues (Fig. 1.1). However, in tissues with dynamic metabolic activity such as the brain or muscle, the vascular density may not be particularly high but perfusion is instead regulated by changes in the vascular diameter and blood flow rates, a process known as hyperemia that can be achieved in seconds after the increased metabolic demand [41–43] – much faster and more efficient than regulating the vascular density by growth or regression of new blood vessels. As such changes in perfusion of a tissue are mainly regulated by vascular dilation/

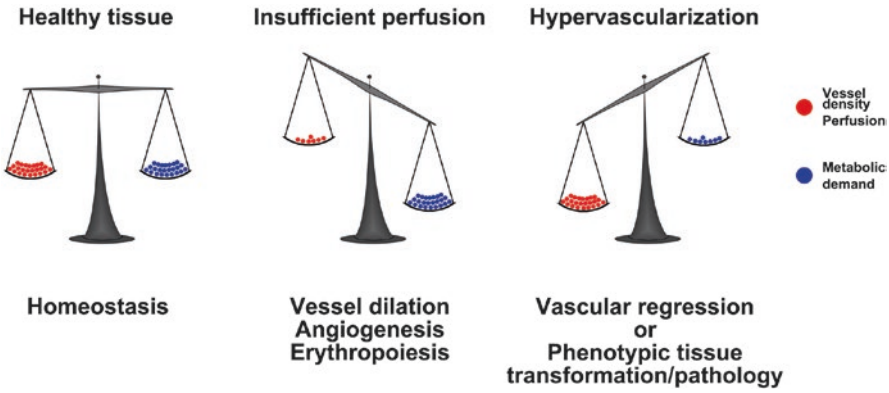

Fig. 1.2 Vascular endowment is balanced by the metabolic needs of the tissue. In healthy tissue the vessel density and perfusion (*red balls*) is balanced according to the metabolic activity of the tissue (*blue balls*; *left image*). Should the metabolism increase (*middle image*), perfusion and oxygen delivery will also increase through vessel dilation, angiogenesis and erythropoiesis. Is however the vascular density or perfusion higher than the needs of the tissue (*right image*), excessive vessels will regress or the metabolic needs of the tissue will increase (phenotypic switch). In diseased tissues, excessive vessels do not regress but rather sustain pathological transformation or growth of the tissue

contraction and hypoxia is the main physiologic mediator of these responses. On the other hand the function of a tissue also changes in response to changes in blood perfusion. For example, forced expansion of the vasculature in adipose tissue by genetic modifications leading to increased expression or local delivery of growth factors by the adipocytes, leads to a phenotypic change in the tissue from energy-storing white to energy consuming brown-like adipose tissue, with a concurrent increase in the metabolic activity of the adipocytes themselves [38, 44–46]. As such, the metabolic activity of a tissue has to be balanced by the blood perfusion, and changes in either the metabolic activity or perfusion parameter drive matching changes in the other, or lead to disease (Fig. 1.2).

Blood vessels, however, are not only a conduit for transport of oxygen, nutrients and metabolic waste products. Many factors important for organizing the functions of various organs to act in concert with each other (i.e. endocrine regulators) also use the vasculature as a route of communication and the vessels play an active role in the signaling process. For example, insulin-dependent and in-dependent glucose uptake by cells in the brain, where there is no passive leakage of glucose across the capillary, is mediated by active transport of glucose across the wall of the blood vessels by Glut1 and Glut4 glucose transporters [47, 48]. Furthermore, hormone receptors are expressed at high levels in the vessels which, in response to hormone stimulation, produce cytokines that mediate some of these hormone-dependent effects on the tissue, thus playing very important roles in endocrine regulation [49, 50]. Also under pathological conditions such as tissue inflammation, the tissue recruit immune cells to fight the inflammatory stimulus (often invaded bacteria) by activating the vessels locally, an activation that lead to the presentation of molecules including ICAM and VCAM, on the inner surface of the vessel which are

recognized by the immune cells and mediate their movement across the vessel and into the tissue specifically at the site of inflammation [49]. These functions are also important for the physiologic trafficking of immune cells to various lymphoid tissues in the organism [51].

The vasculature not only transports chemicals and cells, it is also critical for the thermoregulation by transporting heat [52]. Brown adipose tissue or skeletal muscles are the main sources of heat generation due to their high expression of uncoupling proteins and inherent storage of energy either as fat or glycogen. From here, warm blood is pumped for example through the skin, which is a main tissue responsible for heat/cold sensing [53]. The important role of the blood for maintaining temperature is mirrored in the fact that core body temperature decreases during the inactive period (i.e. night in humans), when the heat generating muscle and brown adipose tissues are less active [54], although many of the other tissues exhibit similar metabolic activity.

Locally, in the tissues, blood vessels also provide key signals for the surrounding cells which are crucial for the specific function. Especially un-differentiated stem- and progenitor cells are known to exist predominantly in so-called vascular nieches, in which these cells make direct contact to endothelial cells, contacts that are crucial for maintaining the undifferentiated state of the cells [55, 56]. As such in tissues as different as the intestine, bone marrow, skin, brain and in tumors, the non-differentiated stem/progenitor cells are specifically found associated with the vasculature [57]. Also other, differentiated cells types require contact with vascular endothelial cells in order to maintain their phenotype. An example of this has recently been demonstrated in adipose tissue, where pericytes loose their identity and transform into adipocytes when their contacts with endothelial cells are disrupted [58]. Similarly, vessels-associated macrophages are primarily of the alternatively differentiated type, whereas non-vessel associated macrophages to a higher extent exhibit the classically activated phenotype [59] indicating that vessel contact may induce or maintain processes required for alternative differentiation and associated functions of macrophages.

During development, the vasculature provide key signals required for organogenesis, regulation of organ size and shape as well as differentiation and specification of cell types in the various organs. The development of the alveoli in the lungs, for example, depends on signals provided by the developing lung vasculature [60]. Should blood vessel growth be inhibited during lung development, this will lead to a failure in the development of alveoli. Also in the kidney, the vasculature provide crucial signals required for differentiation and formation of the glomeruli [61] and in the liver endothelial cells may be important for both the early development of the fetal liver, the structural development of the liver lobules and for the growth and size of the liver both during development and during regeneration [62]. Such regulatory roles of blood vessels also dominate in the regeneration of other organs and tissues, including the skin, in adults [63]. Fin regeneration in fish, for example, is stunted if blood vessel growth is inhibited leading to incomplete regeneration and a smaller fin compared to the size prior to amputation [64]. It is likely that blood vessels do more than simply provide oxygen and nutrients to the developing tissue and as such regulate its size. In zebrafish embryos, oxygenation is not dependent on perfusion