

*COLLOQUIUM SERIES IN
INTEGRATED SYSTEMS PHYSIOLOGY:
FROM MOLECULE TO FUNCTION*

Series Editors: D. Neil Granger & Joey Granger

Angiogenesis

**Thomas H. Adair
Jean-Pierre Montani**



MORGAN & CLAYPOOL LIFE SCIENCES

Angiogenesis

Colloquium Series on Integrated Systems Physiology: from Molecule to Function to Disease

Editors

D. Neil Granger, *Louisiana State University Health Sciences Center–Shreveport*

Joey P. Granger, *University of Mississippi Medical Center*

Physiology is a scientific discipline devoted to understanding the functions of the body. It addresses function at multiple levels, including molecular, cellular, organ, and system. An appreciation of the processes that occur at each level is necessary to understand function in health and the dysfunction associated with disease. Homeostasis and integration are fundamental principles of physiology that account for the relative constancy of organ processes and bodily function even in the face of substantial environmental changes. This constancy results from integrative, cooperative interactions of chemical and electrical signaling processes within and between cells, organs and systems. This eBook series on the broad field of physiology covers the major organ systems from an integrative perspective that addresses the molecular and cellular processes that contribute to homeostasis. Material on pathophysiology is also included throughout the eBooks. The state-of-the-art treatises were produced by leading experts in the field of physiology. Each eBook includes stand-alone information and is intended to be of value to students, scientists, and clinicians in the biomedical sciences. Since physiological concepts are an ever-changing work-in-progress, each contributor will have the opportunity to make periodic updates of the covered material.

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ABSTRACT

Angiogenesis is the growth of blood vessels from the existing vasculature. The field of angiogenesis has grown enormously in the past 30 years, with only 40 papers published in 1980 and nearly 6000 in 2010. Why has there been this explosive growth in angiogenesis research? Angiogenic therapies provide a potential to conquer cancer, heart diseases, and more than 70 of life's most threatening medical conditions. The lives of at least 1 billion people worldwide could be improved with angiogenic therapy, according to the Angiogenesis Foundation. In this little book, we provide a simple approach to understand the essential elements of the angiogenic process, we critique the most powerful angiogenesis assays that are used to discover proangiogenic and antiangiogenic substances, and we provide an in-depth physiological perspective on how angiogenesis is regulated in normal, healthy tissues of the human body. All tissues of the body require a continuous supply of oxygen to burn metabolic substrates that are needed for energy. Oxygen is conducted to these tissues by blood capillaries: more capillaries can improve tissue oxygenation and thus enhance energy production; fewer capillaries can lead to hypoxia and even anoxia in the tissues. This means that angiogenic therapies designed to control the growth and regression of blood capillaries can be used to improve the survival of poorly perfused tissues that are essential to the body (heart, brain, skeletal muscle, etc.) and to rid the body of unwanted tissues (tumors).

KEYWORDS

angiogenesis, angiogenic assays, oxygen, hypoxia, hyperoxia, homeostasis, angiogenic growth factors, vascular endothelial growth factor (VEGF), microcirculation, autoregulation, endothelial cells, adenosine, feedback regulation, lymphatics, lymphangiogenesis

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CHAPTER 1

Overview of Angiogenesis

Angiogenesis is the growth of blood vessels from the existing vasculature. It occurs throughout life in both health and disease, beginning in utero and continuing on through old age. No metabolically active tissue in the body is more than a few hundred micrometers from a blood capillary, which is formed by the process of angiogenesis. Capillaries are needed in all tissues for diffusion exchange of nutrients and metabolites. Changes in metabolic activity lead to proportional changes in angiogenesis and, hence, proportional changes in capillarity. Oxygen plays a pivotal role in this regulation. Hemodynamic factors are critical for survival of vascular networks and for structural adaptations of vessel walls.

Recognition that control of angiogenesis could have therapeutic value has stimulated great interest during the past 40 years. Stimulation of angiogenesis can be therapeutic in ischemic heart disease, peripheral arterial disease, and wound healing. Decreasing or inhibiting angiogenesis can be therapeutic in cancer, ophthalmic conditions, rheumatoid arthritis, and other diseases. Capillaries grow and regress in healthy tissues according to functional demands. Exercise stimulates angiogenesis in skeletal muscle and heart. A lack of exercise leads to capillary regression. Capillaries grow in adipose tissue during weight gain and regress during weight loss. Clearly, angiogenesis occurs throughout life.

1.1 HISTORY

The Scottish anatomist and surgeon John Hunter provided the first recorded scientific insights into the field of angiogenesis. His observations suggested that proportionality between vascularity and metabolic requirements occurs in both health and disease. This belief is summarized in his *Treatise* published in 1794 [1] as follows: “In short, whenever Nature has considerable operations going on, and those are rapid, then we find the vascular system in a proportionable degree enlarged.” Although the term **angiogenesis** does not appear in his writings [1,2], Hunter was the first to recognize that overall regulation of angiogenesis follows a basic law of nature founded by Aristotle [3], which in essence is “**form follows function**.” The modern history of angiogenesis began with the work of Judah **Folkman**, who hypothesized (and published in 1971) that tumor growth is angiogenesis-dependent [4]. Recognition that control of angiogenesis could lead to cancer therapies stimulated intensive

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research in the field, e.g., only two manuscripts dealing with angiogenesis were published in 1970 and over 5200 articles were published in 2009. For detailed histories of angiogenesis, see Refs. [5–11].

1.2 ORIGIN OF BLOOD VESSELS

The cardiovascular system is the first organ system to develop in the embryo [12]. The luminal surface of the circulatory system in contact with blood is a single layer of **endothelial cells**; these are derived from **mesoderm** (Figure 1.1). **Hemangioblasts** differentiate from **mesodermal stem cells** and give rise to **hematopoietic stem cells** and **angioblasts**. Angioblasts are a cell type with potency to differentiate into endothelial cells but have not yet acquired all characteristic markers of endothelial cells. **Vasculogenesis** (Figure 1.2) is the de novo formation of blood vessels from **angioblasts** [12–14]. It occurs in the extraembryonic and intraembryonic tissues of embryos [12,14]. Vasculogenesis is a dynamic process that involves cell–cell and cell–**extracellular matrix** (ECM) interactions directed spatially and temporally by **growth factors** and **morphogens** [14–17]. This process includes differentiation of mesodermal stem cells into angioblasts, growth factor directed migration of angioblasts to form **blood islands** where angioblasts give rise to **endothelial cells** [12–14].

Other types of vascular growth include **arteriogenesis**, **venogenesis**, and **lymphangiogenesis**. The term **neovascularization** means the formation of any blood vessel in the adult regardless of its size or type.

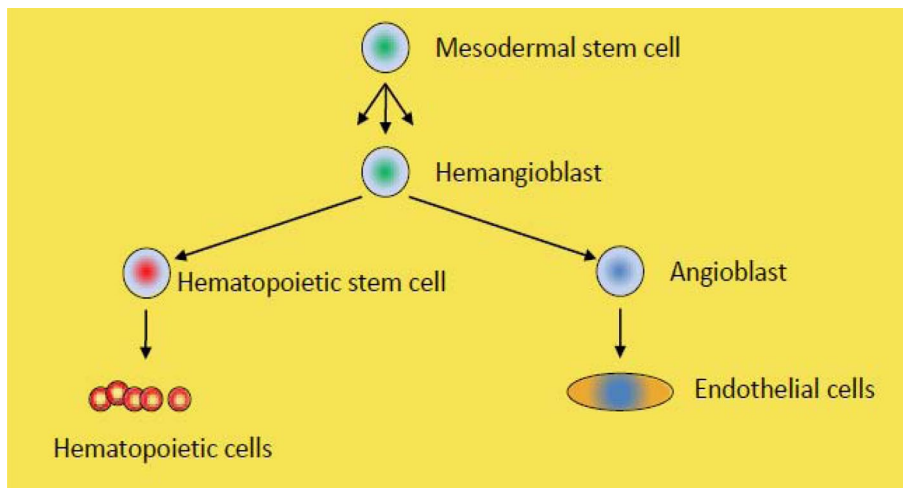


FIGURE 1.1: Origin of endothelial cells and hematopoietic cells [14]. Mesodermal stem cells are the source of hematopoietic stem cells and angioblasts in the developing embryo. The hemangioblast is a precursor to both angioblasts and hematopoietic stem cells. Angioblasts differentiate into endothelial cells. Hematopoietic cells can differentiate into all cell types found in circulating blood

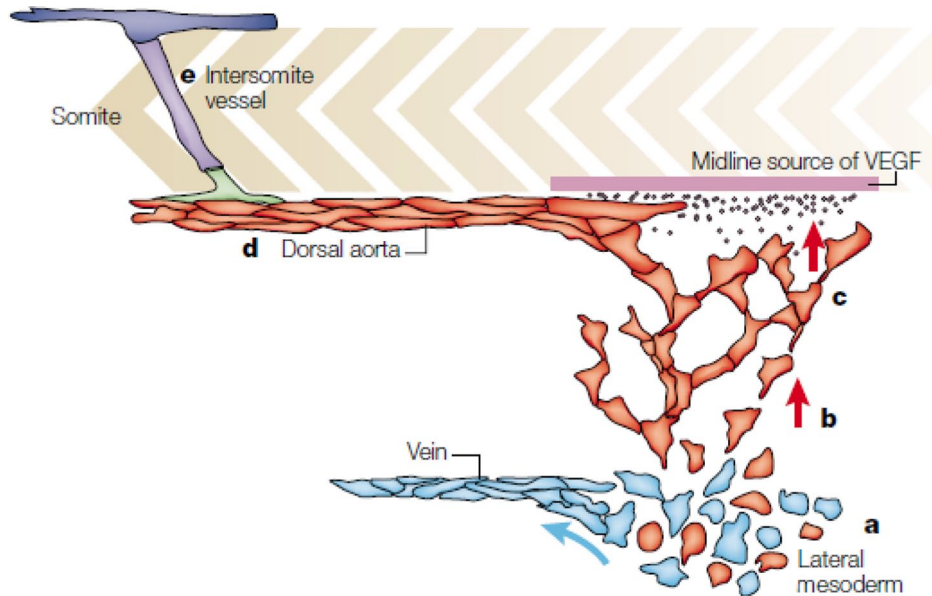


FIGURE 1.2: Vasculogenesis in the vertebrate embryo. (a) Angioblasts derived from lateral mesoderm are committed to become arteries (red) or veins (blue). The cardinal veins assemble from precursor cells (blue) that remain in a lateral position. (b) Artery precursor cells migrate toward a vascular endothelial growth factor type A (**VEGF-A**) stimulus secreted from cells in the midline. (c) The migrating arterial angioblasts align into cords forming a plexus. (d) Arterial angioblasts coalesce forming the dorsal aorta. (e) Intersomite vessels are assembled from three types of endothelial cells with different morphologies indicated as blue, purple, and green. Used with permission from Nature Publishing Group: Hogan (2002) [18].

1.3 THE ANGIOGENIC PROCESS

1.3.1 Types of Angiogenesis

Sprouting angiogenesis and intussusceptive angiogenesis both occur in utero and in adults. Sprouting angiogenesis is better understood having been discovered nearly 200 years ago: intussusceptive angiogenesis was discovered by Burri [19,20] about two decades ago. Figure 1.3 shows the basic morphological events for both types of angiogenesis. As implied by its name, sprouting angiogenesis is characterized by sprouts composed of endothelial cells, which usually grow toward an angiogenic stimulus such as VEGF-A. Sprouting angiogenesis can therefore add blood vessels to portions of tissues previously devoid of blood vessels. On the other hand, intussusceptive angiogenesis involves formation of blood vessels by a splitting process in which elements of interstitial tissues invade existing vessels, forming transvascular tissue pillars that expand. Both types of angiogenesis are thought to occur in virtually all tissues and organs.

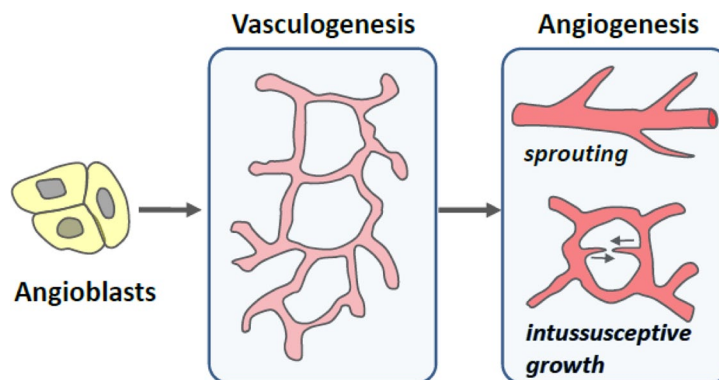


FIGURE 1.3: Basic types of primary vascular growth. Redrawn after Carmeliet and Collen (2000) [21].

1.3.2 Sprouting Angiogenesis

The basic steps of sprouting angiogenesis include enzymatic degradation of capillary basement membrane, endothelial cell (EC) proliferation, directed migration of ECs, tubulogenesis (EC tube formation), vessel fusion, vessel pruning, and pericyte stabilization. Sprouting angiogenesis is initiated in poorly perfused tissues when oxygen sensing mechanisms detect a level of **hypoxia** that demands the formation of new blood vessels to satisfy the metabolic requirements of **parenchymal cells** (Figure 1.4). Most types of parenchymal cells (myocytes, hepatocytes, neurons, astrocytes, etc.) respond to a hypoxic environment by secreting a key proangiogenic growth factor called *vascular endothelial growth factor* (VEGF-A). There does not appear to be redundant growth factor mechanisms that can replace the role of VEGF-A in hypoxia-induced angiogenesis.

An endothelial **tip cell** guides the developing capillary sprout through the ECM toward an angiogenic stimulus such as VEGF-A [22–25]. Long, thin cellular processes on tip cells called **filopodia** secrete large amounts of proteolytic enzymes, which digest a pathway through the ECM for the developing sprout [26,27]. The filopodia of tip cells are heavily endowed with VEGF-A receptors (**VEGFR2**), allowing them to “sense” differences in VEGF-A concentrations and causing them to align with the VEGF-A gradient (Figure 1.5). When a sufficient number of filopodia on a given tip cell have anchored to the substratum, contraction of actin filaments within the filopodia literally pull the tip cell along toward the VEGF-A stimulus. Meanwhile, endothelial **stalk cells** proliferate as they follow behind a tip cell causing the capillary sprout to elongate. Vacuoles develop and coalesce, forming a lumen within a series of stalk cells. These stalk cells become the trunk of the newly formed capillary. When the tip cells of two or more capillary sprouts converge at the source of VEGF-A secretion, the tip cells fuse together creating a continuous lumen through which oxygenated blood can flow. When the local tissues receive adequate amounts of oxygen, VEGF-A levels

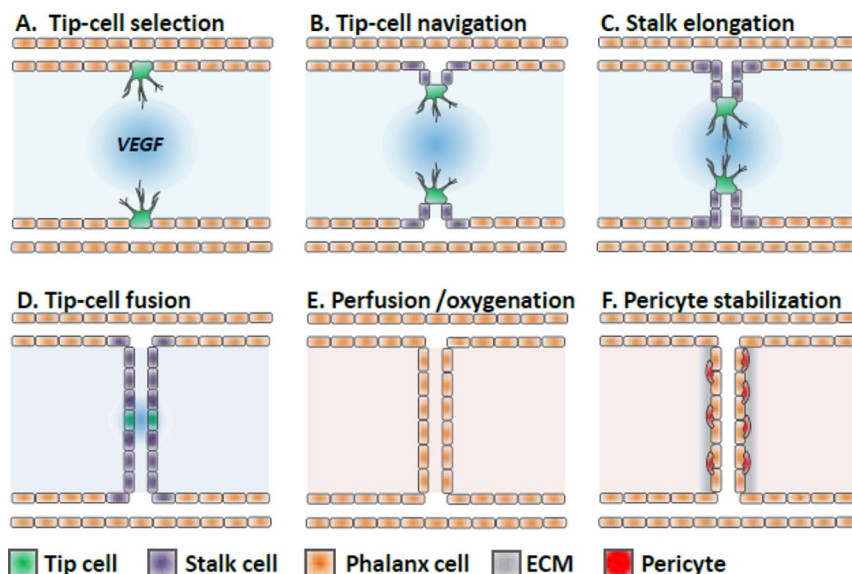


FIGURE 1.4: VEGF-A directed capillary growth to poorly perfused tissues. (A) Endothelial cells exposed to the highest VEGF-A concentration become tip cells (green). Hypoxic tissue is indicated by the circular blue fade. (B) The tip cells lead the developing sprout by extending numerous filopodia. (C) The developing spout elongates by proliferation of endothelial stalk cells (purple) that trail behind the tip cell. (D) The tip cells from two developing sprouts fuse and create a lumen. (E) Blood flowing through the new capillary oxygenates the tissues, thus reducing the secretion of VEGF-A. (F) The newly developed capillary is stabilized by pericyte recruitment (red), deposition of ECM (gray), shear stress and other mechanical forces associated with blood flow and blood pressure. Redrawn after Carmeliet et al. (2009) [24].

return to near normal. Maturation and stabilization of the capillary requires recruitment of [pericytes](#) and deposition of ECM along with [shear stress](#) and other mechanical signals [28].

Delta-Notch signaling is a key component of sprout formation (Figure 1.5). It is a cell–cell signaling system in which the ligand, [Delta-like-4](#) (Dll4) mates with its [notch receptor](#) on neighboring cells. Both the receptor and ligand is cell bound and thus act only through cell–cell contact. VEGF-A induces Dll4 production by tip cells, which leads to activation of notch receptors in stalk cells. Notch receptor activation suppresses VEGFR2 production in stalk cells, which dampens migratory behavior compared with that of tip cells. Hence, endothelial cells exposed to the highest VEGF-A concentration are most likely to become tip cells [24,25,30]. Although tip cells are exposed to the highest VEGF-A concentration, their rate of proliferation is far less compared with that of stalk cells.

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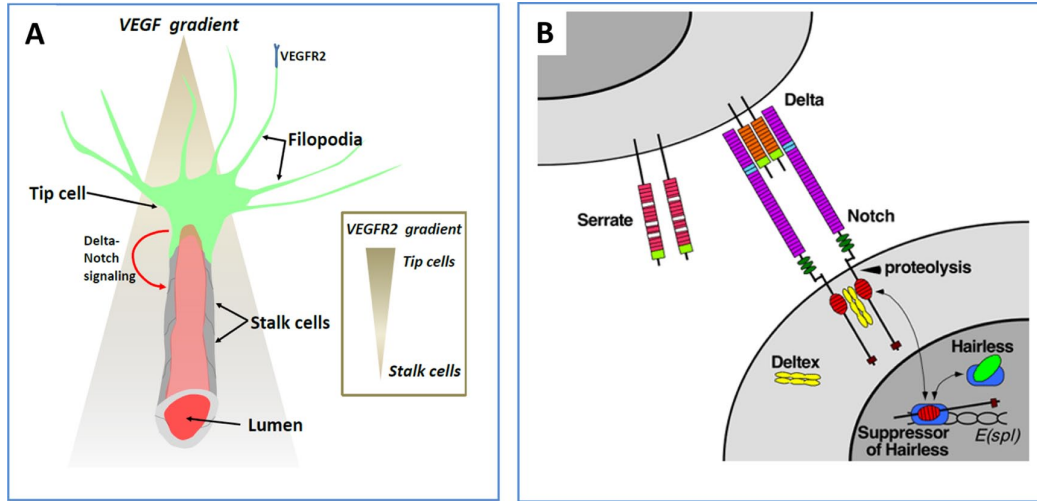


FIGURE 1.5: Microanatomy of a capillary sprout and tip cell selection. (A) An interstitial gradient for VEGF-A and an endothelial cell gradient for VEGFR2 are shown. Tip cell migration is thought to depend upon the VEGF-A gradient and stalk cell proliferation is thought to be regulated by the VEGF-A concentration. Redrawn after Carmeliet and Tessier-Lavigne (2005) [29]. (B) Delta-Notch signaling is critical for tip cell selection. Activation of notch receptors on stalk cells induces proteolytic cleavage and release of the intracellular domain, which enters the nucleus and decreases gene expression of VEGFR2. National Institutes of Health, public domain image.

Not all aspects of the Delta-Notch signaling pathway are fully understood, but it is clear that production of a normal vasculature is heavily dependent upon the concentration of VEGF-A in the tissues. A 50% reduction of VEGF-A expression is lethal embryonically because of vascular defects [31,32], and excess VEGF-A in tumors induces overproduction of tip cells leading to a disorganized vasculature [33]. This critical dependence on physiological concentrations of VEGF-A for construction of viable blood vessels might help explain why attempts to induce angiogenesis in poorly perfused tissues with VEGF-A administration and gene therapy have not been highly successful.

1.3.3 Intussusceptive Angiogenesis

Intussusceptive angiogenesis is also called *splitting angiogenesis* because the vessel wall extends into the lumen causing a single vessel to split in two. This type of angiogenesis is thought to be fast and efficient compared with sprouting angiogenesis because, initially, it only requires reorganization of existing endothelial cells and does not rely on immediate endothelial proliferation or migration. Intussusceptive angiogenesis occurs throughout life but plays a prominent role in vascular develop-

ment in embryos where growth is fast and resources are limited [34–36]. However, intussusception mainly causes new capillaries to develop where capillaries already exist.

Evidence for the occurrence of intussusceptive angiogenesis is based upon the presence of transcapillary tissue pillars (Figure 1.6). Identification of tissue pillars requires scanning electron micrographs of vascular casts or three-dimensional reconstruction of serial micrographs. This type of angiogenesis was discovered in postnatal lungs of rats and humans [19,20], but it also occurs in many other tissues and organs, especially in capillary networks that abut an epithelial surface, e.g., choroid of the eye, vascular baskets around glands, intestinal mucosa, kidney, ovary, and uterus [37,38]. It also occurs in skeletal muscle, heart, and brain. In addition to forming new capillary structures, intussusceptive growth plays a major role in the formation of artery and vein bifurcations as well as pruning of larger microvessels.

The control of intussusceptive angiogenesis is poorly understood compared with sprouting angiogenesis. This difference is only partly due to its recent discovery in 1986 [20]. A rate-limiting step in intussusceptive growth research can be pinned to the laborious methods required to prove its presence, which, again, involve determining the frequency of tissue pillars from scanning electron micrographs of vascular casts. However, it is known that intussusceptive angiogenesis can be stimulated in the chick **chorioallantoic membrane** (CAM) with application of VEGF-A (Figure 1.7), and there is little doubt that many growth factors and signaling systems are involved [34,37]. Mechanical stresses related to increases in blood flow can initiate intussusceptive growth in some high flow regions of the circulation, as discussed in Chapter 4 [34,35].

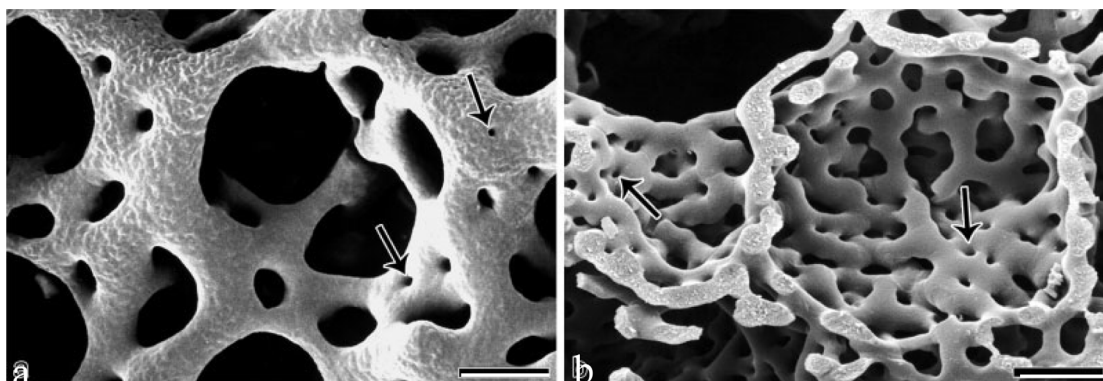


FIGURE 1.6: Scanning electron micrographs of Mercor casts. (a) Fetal chicken lung microvasculature. (b) Rat lung microvasculature at postnatal day 44. The small holes indicated by arrows have diameters of about 2 μm. The holes correspond to tissue pillars that extend across the capillary lumina. Scale bars: (a) 12 and (b) 20 μm. Used with permission from Wiley-Blackwell: Djonov, Kurz, and Burri (2003) [35].

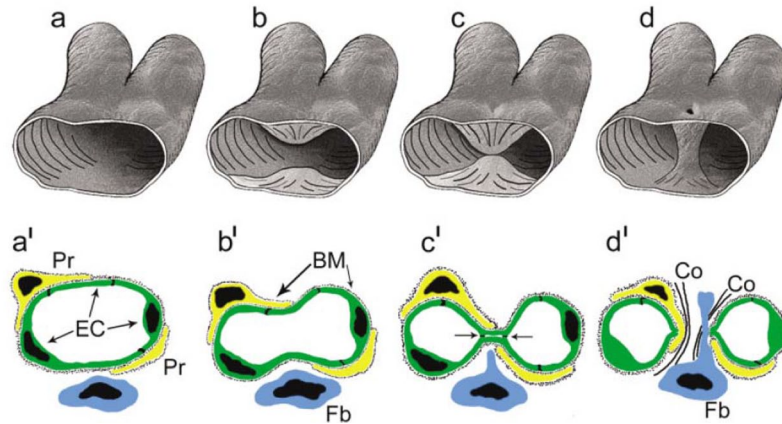


FIGURE 1.7: Intussusceptive angiogenesis in three dimensions (a–d) and two dimensions (a'–d'). (a,b, a',b') The process begins with protrusion of opposing endothelial cells into the capillary lumen. (c,c') An interendothelial contact is established and endothelial junctions are reorganized. (d,d') The endothelial (EC) bilayer and basement membranes (BM) are perforated centrally allowing growth factors to enter. Fibroblasts (Fb) and pericytes (Pr) migrate into the site of perforation where they produce collagen fibrils (Co) and other components of ECM forming a tissue pillar. Used with permission from Wiley-Blackwell: Djonov, Kurz, and Burri (2003) [35].

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CHAPTER 2

Angiogenesis Assays

The most significant advancements in angiogenesis in the past 25 years have been the discovery of proangiogenic and antiangiogenic molecules. The development of angiogenesis assays has been essential to the discovery of these molecules. In vitro assays have been expeditious and quantitative but should be viewed as first approximations that need to be confirmed in vivo. In vivo tests are more time-consuming and difficult to quantitate, but because of the complex interactions between multiple cell types necessary to form functional blood vessels, all in vitro findings need to be confirmed in the intact animal.

2.1 IN VITRO ASSAYS

2.1.1 Endothelial Cells Are Heterogeneous

Most **endothelial cell** assays utilize human umbilical vein endothelial cells (HUVECs) or bovine aortic endothelial cells (BAECs) not because these cells are good representatives of vascular endothelial cells in vivo, but because they are relatively easy to harvest from large blood vessels. Endothelial cells are heterogeneous [39–43]: there are differences among endothelial cells from large and small blood vessels, arteries, and veins, species differences, organ differences, differences between host and tumor, and even differences within a given organ. Also, endothelial cells used in laboratories are virtually always in a proliferative state rather than the normal quiescent state of the established vasculature in the intact animal. Even most “primary cultures” of endothelial cells require extensive proliferation to obtain enough cells for an experiment with the assumption that they retain their normal in vivo physiological characteristics. This assumption is often incorrect. It is well-known that cells in vitro can both gain and lose attributes compared with parent cells in intact animals.

2.1.2 In Vitro Conditions Rarely Reflect In Vivo Environment

Another issue is the environmental conditions in which endothelial cells are cultured. Endothelial cells in vivo are exposed to shear stress and other hemodynamic forces that activate multiple signaling pathways. Endothelial cells in vitro are usually cultured in room air (21% oxygen), which is hyperoxic compared with the in vivo oxygen tension, especially in the microcirculation. Two- and three-dimensional scaffolds using Matrigel, collagen, or fibrin are used to simulate the normal

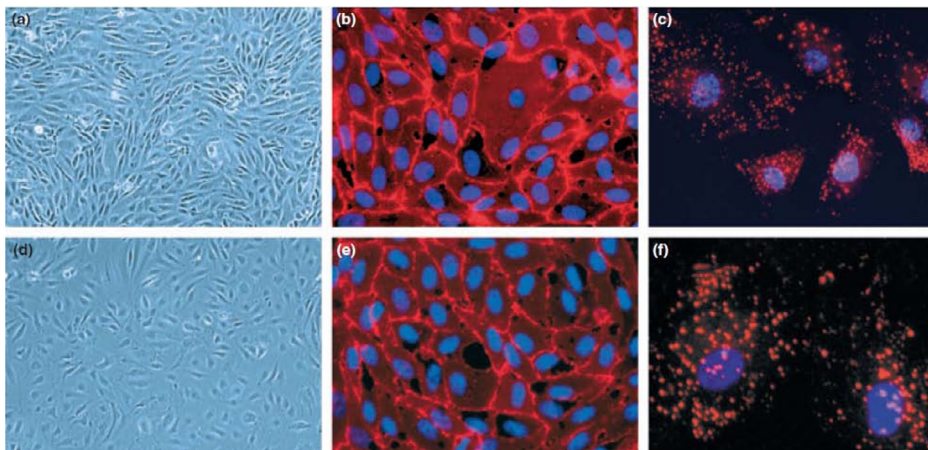


FIGURE 2.1: (a–c) Human umbilical vein endothelial cells (HUVEC) and (d–f) human dermal microvascular endothelial cells (HuDMEC). (a, d) Phase contrast microscopy. (b, e) CD31 or (c, f) von Willebrand factor staining: nuclei are counterstained with DAPI. Photographs courtesy of Promocell GmbH, Heidelberg, Germany. Used with permission from Wiley-Blackwell: Staton et al. (2009) [42].

extracellular matrix, but it is clear that complex interactions between endothelial cells and their *in vivo* physical environment cannot be fully simulated in culture. Also, *in vivo* interactions between endothelial cells and other cell types (smooth muscle cells, pericytes, fibroblasts, macrophages, etc.) are difficult to simulate *in vitro*. For these reasons, *in vitro* angiogenesis assays should be viewed as a starting point rather than an endpoint for discovery, depending on the purpose of the experiment (Figure 2.1).

2.1.3 Endothelial Cell Proliferation Assays

Proliferation of endothelial cells is needed for developing capillaries in the intact animal. The actions of proangiogenic and antiangiogenic molecules on proliferation can be assessed by direct cell counts, DNA synthesis, or metabolic activity [39–42,44]. For testing potential proangiogenic molecules, it is often necessary to reduce the rate of proliferation by decreasing serum levels in culture media, and it is usually more effective to test antiangiogenic molecules on cells that have a substantial rate of proliferation.

The rate of cell proliferation can be determined by counting cells at 24-h intervals after seeding multiple cultures with a defined number of cells (Figure 2.2). Cells can be counted using a hemocytometer and light microscope or an electronic Coulter counter or similar device. Cell proliferation is often determined using a colorimetric method (MTT assay) in which mitochondrial enzymes

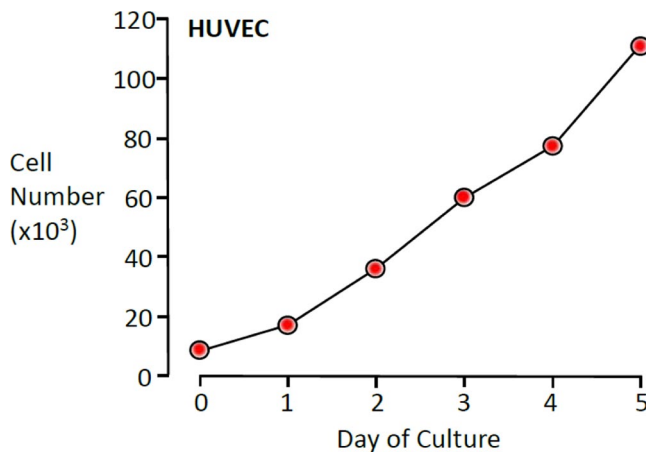


FIGURE 2.2: Typical growth curve for HUVECs in culture media containing 10% fetal bovine serum (FBS). Media were changed daily. Cells were counted using a Coulter counter. Redrawn after Lee (2006) [44].

reduce MTT to formazan dyes in proportion to cell number. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazole that is reduced to purple formazan in living cells: absorbance is read by a spectrophotometer. DNA synthesis is often used to reflect cell proliferation. With the commonly used [³H]thymidine incorporation method, the amount of radioactivity in cells is proportional to the amount of newly synthesized DNA. A similar but nonradioactive method utilizes bromodeoxyuridine (BrdU), which competes with thymidine for incorporation into DNA. Despite their popularity, these latter methods are not fully reliable. Changes in metabolic rates of individual cells and/or compounds that affect mitochondrial enzyme activity can cause gross miscalculations of cell proliferation with the MMT assay, and several investigators have found that DNA synthesis can occur without a concomitant increase in cell proliferation [45–47]. Because of the above problems, it is best to use more than one method for determining cell proliferation. But clearly, the most reliable method is direct counting of individual cells.

2.1.4 Endothelial Cell Migration Assays

In sprouting angiogenesis, endothelial cells degrade the basement membrane and migrate along chemical gradients established by proangiogenic growth factors. The transfilter assay [39,40,48] (Figure 2.3) is used frequently to assess endothelial cell migration: it is a modification of the classical Boyden chamber assay [49]. The method is highly sensitive to low levels of chemotactic factors and highly reproducible compared with other migration assays. Special provisions can be used to differentiate

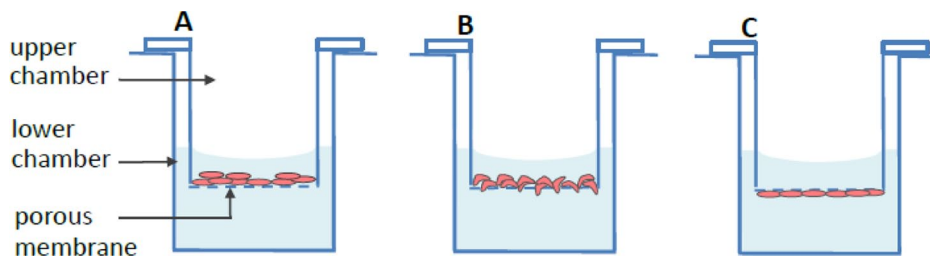


FIGURE 2.3: Transfilter migration assay. (A) Endothelial cells are placed in the upper chamber where they rest upon the filter. (B) The filter pores are small enough ($\sim 8 \mu\text{m}$) to allow passage of actively migrating cells. A chemotactic test substance placed in the lower chamber can induce cells to migrate through the pores and into the lower chamber. (C) Cells that fail to migrate are removed from the upper side of the filter with a cotton swab: migrated cells are fixed, stained, and counted by eye. The entire assay can usually be completed in a few hours. Redrawn after Polytarchou et al. (2006) [48].

between **chemokinesis** and **chemotaxis** (Figure 2.4). There are many other migration assays including the under-agarose assay, wound healing assay, Teflon fence assay, phagokinetic track assay, and others described elsewhere [39–42,48].

2.1.5 Endothelial Tube Formation Assays

Once the endothelial cells have proliferated and migrated toward a proangiogenic growth factor stimulus, they must form tubes with lumens to conduct the flow of blood. Tube formation is often

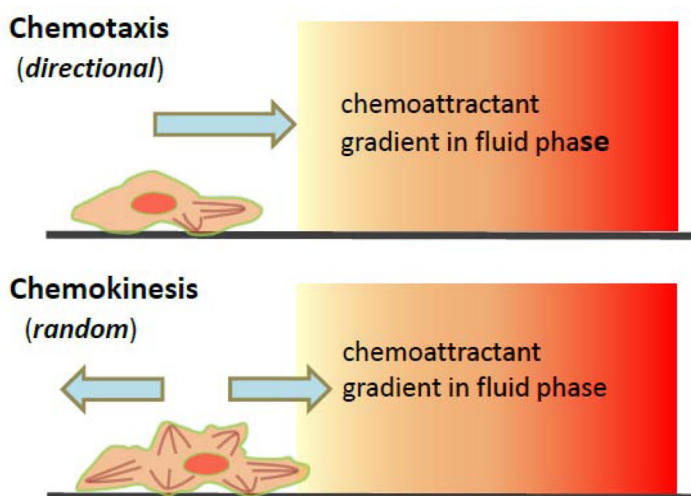


FIGURE 2.4: The basic difference between **chemotaxis** and **chemokinesis** is shown.

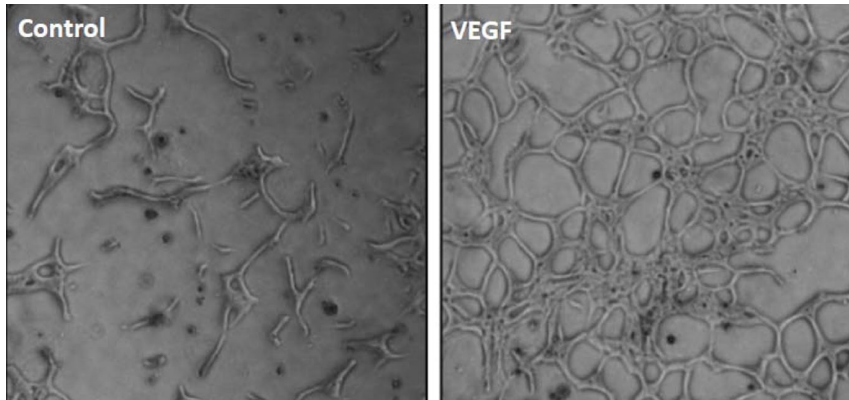


FIGURE 2.5: Matrigel tube formation assay. BAECs were suspended in diluted Matrigel for an overnight incubation and then subjected to a media change containing VEGF-A (10 ng/mL). Capillary-like structures presumed to have a lumen are apparent after three days of treatment. Used with permission from Elsevier: Goodwin (2007) [39].

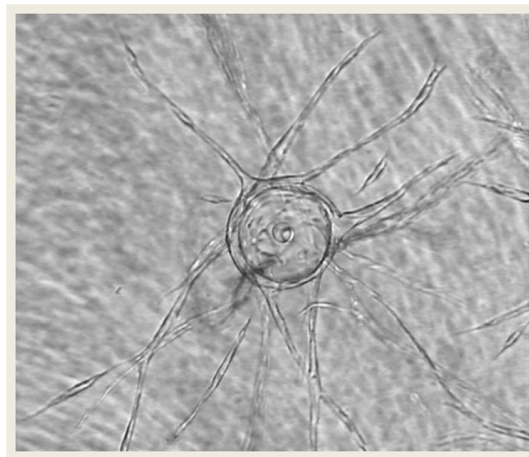


FIGURE 2.6: The fibrin gel bead assay recapitulates key steps of the early angiogenic process and, importantly, the vessels display patent intercellular lumens surrounded by polarized endothelial cells. Cytodex microcarriers (beads) coated with HUVECs are embedded into a fibrin gel, and fibroblasts layered on the gel surface provide soluble factors that promote endothelial sprouting from the surface of the beads. A how-to video of the method can be viewed at the following URL: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2570172/?tool=pubmed> [51]. Photomicrograph courtesy of Dr. Christopher C.W. Hughes.

called *tubulogenesis*. It represents the later stages of the angiogenic process in which endothelial cells differentiate. Currently, the most popular tube formation assays involve plating human umbilical vein endothelial cells (HUVECs) or bovine aortic endothelial cells (BAECs) with Matrigel (Figure 2.5) [39,50]. A potential issue with the Matrigel assay is that cells of nonendothelial origin (e.g., fibroblasts, carcinoma cells, glioblastoma cells) have been shown to form “tubes,” which raises the question of whether tubes with actual lumens are formed or whether cords of cells without lumens might be also be formed in this assay [50]. In addition to the Matrigel assay (a two-dimensional assay), there are three-dimensional assays such as the fibrin gel bead assay that assesses additional components of the angiogenic process (proliferation and migration) (Figure 2.6).

2.1.6 Rat and Mouse Aortic Ring Assay

The aortic ring angiogenesis assay is used widely in angiogenesis research [52–54] (Figure 2.7). It is highly reliable and reproducible. The aorta is removed, cut into ~1-mm sections, and embedded into a collagen or fibrin matrix. In serum-free media, the microvessels begin sprouting from rat explants by day 3 in culture as shown (Figure 2.7). The vascular outgrowths are very similar to normal blood vessels and are composed of the same cell types. The sprouting microvessels interact closely with resident macrophages, pericytes, and fibroblasts in an orderly sequence that emulates angiogenesis in the intact animal. The endothelial cells have not been preselected by passaging and are thus in a quiescent state similar to that of the intact animal. However, unlike skeletal and car-

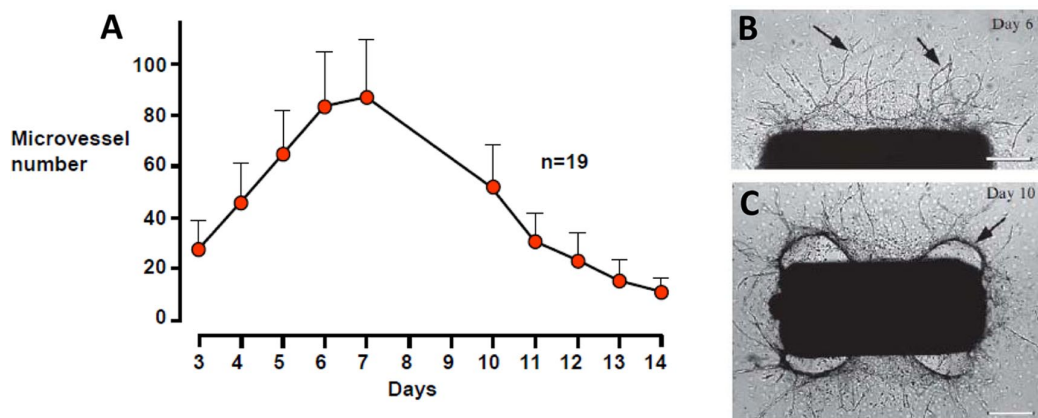


FIGURE 2.7: Rat aortic ring angiogenesis assay. (A) The number of microvessels increases progressively during the first week in culture: microvessels deteriorate during the second week (T.H. Adair, unpublished data). Arrows show microvessels at day 6 (B) and halos of collagen lysis at day 10 (C). Scale bar = 400 μ m. Photographs used with permission from Elsevier: Aplin et al. (2008) [53].

diac muscle organ assays [55,56], microvessel growth is not stimulated significantly by exposure to a hypoxic environment, which likely reflects in vivo adaptation to arterial oxygen tension. Other in vitro angiogenesis assays that incorporate all angiogenic functions (matrix degradation, migration, proliferation, tube formation) include the embryoid assay, mouse metatarsal assay, and others [39].

2.2 IN VIVO ASSAYS

Most investigators in the field of angiogenesis place greater emphasis on research results from intact animals. Complex interactions among multiple cell types, the extracellular matrix, hemodynamic factors, and metabolic factors are difficult, if not impossible, to duplicate in vitro. There is little doubt that angiogenesis associated with normal bodily functions (such as exercise) can provide superior insights into the overall regulation of angiogenesis; however, a number of in vivo assay systems have been developed for testing putative proangiogenic and antiangiogenic molecules. These assays include the corneal angiogenesis assay, chick chorioallantoic membrane (CAM) assay, Matrigel plug assay, and others [5,40–42].

2.2.1 Corneal Angiogenesis Assay

The cornea is the only tissue of the body that is both avascular and transparent. These unique anatomical features make the cornea ideal for observation of angiogenesis in mice, rats, and rabbits (Figure 2.8). Slow-release polymer pellets or sponges containing proangiogenic molecules (VEGF-A,

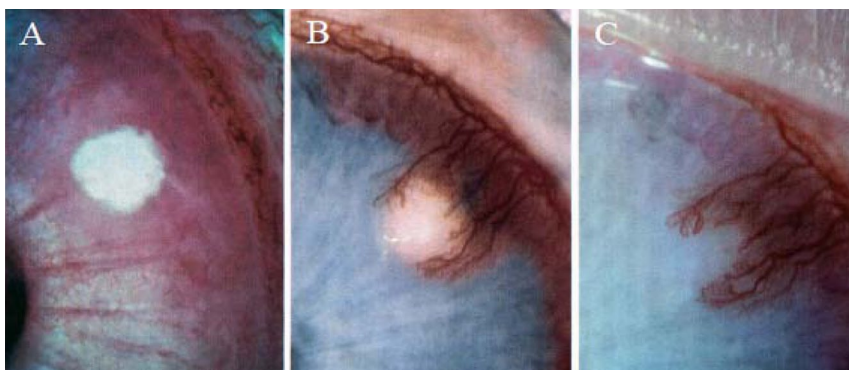


FIGURE 2.8: (A) A sustained-release polymer pellet implanted into the cornea of a rabbit eye does not cause inflammation or edema in this example. (B) A pellet containing a proangiogenic molecule causes new vessels to grow from the limbal edge of the cornea between the lamellar layers of the cornea. (C) The pellet can be removed through a small incision. Blood vessels will have regressed completely by about 10 weeks. Used with permission from Springer Science+Business Media: Folkman (2008) [5].

FGF2) or tumor cells are implanted into stromal pockets created surgically. The ingrowth of new vessels from the peripheral limbal vasculature can be monitored daily, allowing rates of angiogenesis to be determined. Putative antiangiogenic molecules can then be administered orally or parenterally to determine their effect on the rate of local angiogenesis in the cornea.

2.2.2 Chick Chorioallantoic Membrane (CAM) Angiogenesis Assay

The CAM is a gas exchange membrane that lies directly under the egg shell of all avian species. The CAM is highly vascular, readily accessible, and chicken eggs are inexpensive. Large numbers of eggs can be prepared for the CAM assay in a relatively short time and results can be quantitated rapidly with minimal equipment allowing large-scale screening. Although the CAM assay can be performed in ovo by removing a small section of the egg shell, many investigators have transferred the developing chick embryo to a plastic culture disk or other container after about 3 days of incubation: this permits far better visualization of the CAM vasculature, permits serial measurements to be made with ease, and allows control and experimental molecules to be tested on the same CAM (Figure 2.9). Many different delivery methods have been used: slow-release polymer pellets, Silastic rings into which test molecules are pipetted, sponges, filter discs, and so forth [40–42,57]. Quantitation of angiogenic and antiangiogenic responses are often performed at low magnification, making it difficult to differentiate between actual growth of blood vessels and dilation of existing vasculature. Although highly quantitative methods have been developed, these are often laborious and time consuming [58].

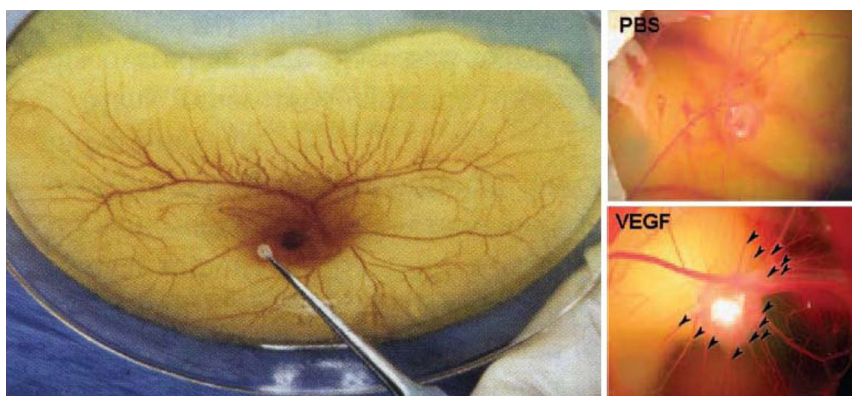


FIGURE 2.9: (left) Placement of a test substance on the shell-less chick embryo chorioallantoic membrane (CAM). (right) VEGF-A induces angiogenesis in the CAM vasculature compared with a PBS control. Used with permission from Springer Science+Business Media: Folkman (2008) [5] and Nature Publishing Group: Dai et al. (2009) [59].

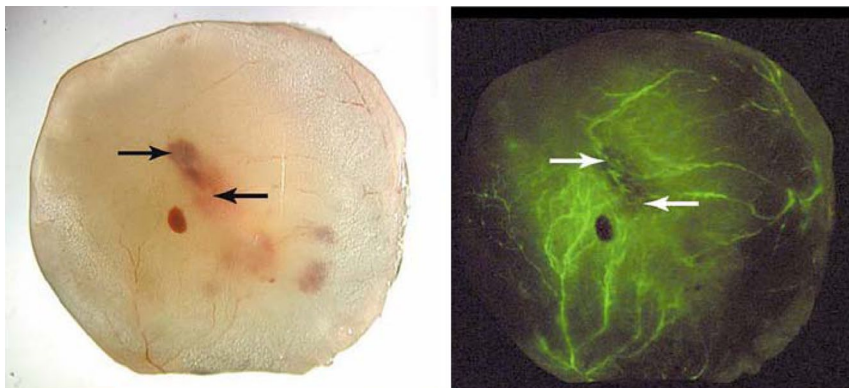


FIGURE 2.10: The angiogenic response to tumor tissue implanted into a Matrigel plug in a mouse is shown. Following plug removal and fixation, the vasculature can be seen via (left) phage illumination and (right) UV illumination following intravenous administration of dextran-FITC. Fluorescence can be quantitated using standard programs such as Photoshop. Used with permission from Springer Science+Business Media: Akhtar et al. (2002) [60].

2.2.3 Matrigel Plug Assay

The Matrigel plug assay has become the method of choice for testing angiogenesis in vivo [40,60]. Matrigel is the trade name of a gelatinous protein mixture harvested from mouse sarcoma cells. The mixture resembles the extracellular matrix and contains multiple proangiogenic growth factors. Cold Matrigel administered subcutaneously solidifies within about 30 minutes. A sponge, slow-release pellet, or other delivery system containing a test substance can then be implanted in the Matrigel plug through a small incision. The Matrigel plug is removed several days later and imaged. Dextran-FITC can be administered intravenously before plug removal to facilitate visualization of blood vessels as shown (Figure 2.10). Antiangiogenic molecules administered systemically can be tested with this method.

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CHAPTER 3

Regulation: Metabolic Factors

The regulation of blood and lymph vessel growth adheres to a law of nature first described by Aristotle [3], which in essence is “**form follows function**.” In heart, brain, skeletal muscle, and other tissues where blood vessels have primarily a nutritive function, the vasculature grows and regresses in accordance with the metabolic needs of the tissues. Oxygen plays a central role in the control of angiogenesis in these tissues: stimulation of growth occurs under hypoxic conditions and **microvascular rarefaction** occurs when the tissues are overoxygenated.

3.1 CAPILLARY GROWTH IS PROPORTIONAL TO METABOLIC ACTIVITY

The relation between capillary growth and metabolic activity has been studied most extensively in skeletal muscles. Muscle fibers and capillaries are arranged in parallel, which facilitates measurements of the capillary network. Krogh [61,62] introduced a method to characterize the structure of the capillary network, which involves counting the number of capillary profiles per unit area of muscle cross section. This classical parameter, commonly referred to as *capillary density*, still provides a reasonable estimate of the size of a capillary bed in skeletal muscle because of the above-mentioned special geometry. Using this method, Krogh [61] studied the skeletal muscles of horse, dog, and guinea pig and concluded that the density of capillaries in the muscles is proportional to the basal metabolic rate of the animal. This conclusion has been substantiated by many studies [63–65]. A more rigorous relationship between capillarity (capillary length density) and metabolic requirements (mitochondrial volume density) can be determined using the principles of **stereology** (Figure 3.1).

This relationship between oxidative capacity and capillarity occurs at the cellular level. Capillary density associated with highly oxidative muscle fibers is about twice that of glycolytic fibers in the same skeletal muscle [74]. Although early measurements established proportionality between capillarity and metabolic activity in heart and skeletal muscle, a similar relationship is evident in various structures in the brain, gastrointestinal tract, smooth muscle, glandular structures, and other tissues where the vasculature has a prominent role in diffusion exchange of nutrients and metabolites.

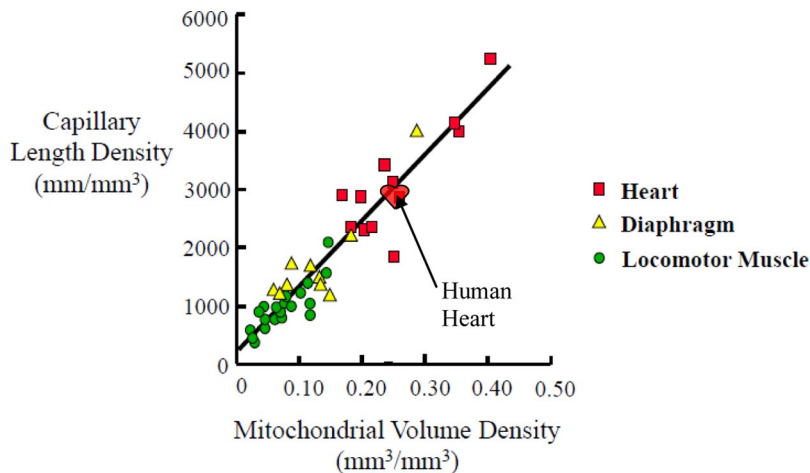


FIGURE 3.1: Capillary length density and mitochondrial volume density are shown for 13 mammalian species spanning more than 5 orders of magnitude in body size. Capillary length density is a stereological parameter representing a statistical estimate of total capillary length per unit volume of reference tissue (in this case, muscle fiber volume) that includes vessel tortuosity [66–70]. Mitochondrial volume density refers to mitochondrial volume per unit volume of this same reference tissue: it is a structural measure of the oxidative capacity of a tissue [71,72]. A generally linear relationship is evident. Note that heart (red) with its relatively high oxidative capacity has a correspondingly high capillary length density. Capillary length density of human heart is shown [73]. Redrawn after Adair et al. (1990) [63] and Hoppeler and Kayar (1988) [64].

3.2 INCREASING METABOLIC ACTIVITY STIMULATES BLOOD VESSEL GROWTH

A dynamic relationship between capillarity and metabolic activity is ubiquitous in tissues where the vasculature has mainly a nutritive function. In these tissues, which include skeletal muscle, heart, brain, and others, primary changes in metabolic activity (or oxygen availability) invariably lead to secondary proportional changes in blood vessel growth or regression. Recognition that increased metabolic activity can promote angiogenesis originates from studies of endurance exercise training. Exercise training increases oxidative capacity and stimulates angiogenesis in skeletal muscle [75–80]. Chronic stimulation of a motor nerve to a glycolytic muscle at a slow frequency characteristic of oxidative muscle converts glycolytic fibers to oxidative fibers and causes extensive angiogenesis (Figure 3.2) [81,82] as well as growth of all arteries and veins [83]. Cross-innervation studies show similar results [84].

Other examples in which increased metabolic activity stimulates angiogenesis include non-shivering thermogenesis in which muscle metabolism and angiogenesis increase without actual contraction of the muscle [85–87] and prolonged administration of thyroid hormone, which increases

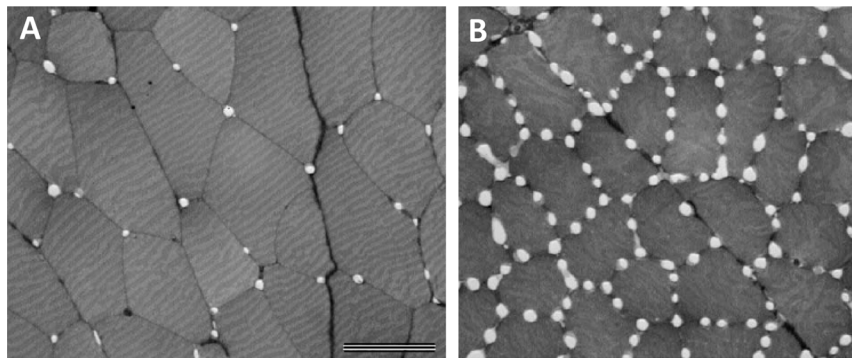


FIGURE 3.2: Chronic increases in muscular activity stimulate angiogenesis in rat skeletal muscle. Control (A) and stimulated (B) anterior tibialis muscles were taken from contralateral limbs of the same rat. The peroneal motor nerve was stimulated for 2-h periods followed by 4-h periods of rest for 30 days. Stimulation parameters were 10-Hz, 300- μ s square wave pulses, and 3–5 V. The 30 days of stimulation converted the predominantly fast-twitch, glycolytic anterior tibialis muscle (A) to a predominantly slow-twitch, oxidative muscle (B) with increased capillarity and decreased fiber diameter as shown. The muscles were perfused-fixed with glutaraldehyde at physiological blood pressures. These are 1- μ m-thick plastic sections taken from the same location of each muscle. Bar = 50 μ m (T.H. Adair, unpublished data).

oxidative capacity and stimulates angiogenesis in skeletal muscle [88,89], heart [90,91], various structures in brain [92], and bone [93].

Imbalances between metabolic requirements and vascularity can also result from exposing animals to different oxygen atmospheres. For example, subjecting an animal to a hypoxic environment can limit oxygen supply to some tissues even though metabolic requirements have not changed. Prolonged exposure of the developing chick embryo to a hypoxic environment stimulates microvessel growth in the chorioallantoic membrane (CAM) [58] as well as growth of major arteries within the embryo itself [63] (Figure 3.3). Opposite results occur when embryos are exposed to a hyperoxic environment as shown.

Many other studies have shown that long-term exposure to a hypoxic environment can promote angiogenesis in avian embryos [94–99]. Hypobaric hypoxia can stimulate angiogenesis in various structures in the rodent brain [100–103]. Exposing infants (or newborns of other animal species) to a hyperoxic environment inhibits normal development of the retinal vascular bed: neovascularization follows with subsequent exposure to room air [104–110]. The peripheral avascular retina is assumed to be hypoxic when animals are transferred from hyperoxic to normoxic environments because the choroidal vasculature (the only oxygen supply) does not autoregulate with infinite gain, which is true for blood flow control in most vascular beds. Subjecting experimental wounds to a hypoxic environment accelerates angiogenesis in the wound [111]. Patients with peripheral arterial

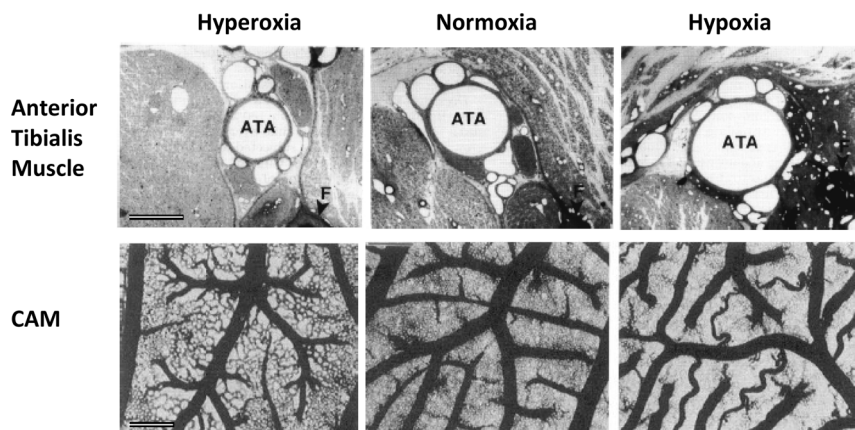


FIGURE 3.3: Chronic exposure to a hypoxic environment (12% oxygen) stimulates diameter growth of the anterior tibialis artery (ATA) as well as blood vessel growth in the chorioallantoic membrane (CAM) [58,63]. Exposure to a hyperoxic environment (70% oxygen) decreases growth of the CAM vasculature and ATA, compared with growth in a normoxic environment (21% oxygen). (lower right) Tortuous vessels in the CAM are often observed following incubation in 12% oxygen. In these studies, chick embryos were incubated in the different oxygen atmospheres from days 7 to 14 of incubation. The body of the chick embryo was perfused-fixed with glutaraldehyde at physiological pressures, embedded in plastic, and 1- μ m sections were cut with glass knives and stained with toluidine blue. CAMs were filled with colloidal carbon in albumin solution (10% final concentration) and fixed with glutaraldehyde. Bars = 200 μ m.

disease have increased capillary density and increased mitochondrial enzyme activity [112–117] that can be further increased with training [118,119]. However, prolonged exposure to a hypoxic environment (often called hypoxic-hypoxia) does not appear to stimulate angiogenesis in skeletal muscles of adult mammals [120–122]. Early studies showing hypoxia-induced increases in capillary density [120–124] can be explained by hypoxia-induced decreases in muscle fiber diameter [120,121,124]. A decrease in fiber diameter can increase capillary density without actual growth of capillaries. Recent studies suggest strongly that hypoxic-hypoxia does not stimulate angiogenesis in human skeletal muscle under resting conditions, but can have a limited role in promoting angiogenesis during training [125]. Studies performed in rats support a role for hypoxic-hypoxia in augmenting angiogenesis during treadmill exercise training [126].

3.3 DECREASING METABOLIC ACTIVITY CAUSES VASCULAR REGRESSION

Capillary rarefaction (also called *capillary dropout*) is a well-known consequence of **disuse atrophy** and **tenotomy** in skeletal muscle. Virtually any perturbation that causes a long-term decrease in mus-

cular activity is followed by deterioration of the muscle vascular system. Ultrastructural studies provide clear evidence of capillary degeneration in models of muscle disuse [127,128]. In each of the following examples, a decrease in muscular activity was followed by a decrease in the ratio of capillaries to muscle fibers (C/F): experimental tenotomy in rats [129], tenotomy by spontaneous rupture in humans [130], immobilization in a plaster cast [129], application of tetrodotoxin to a motor nerve [131], hind limb suspension in rats [132,133], various myopathies in humans [134,135], and weightlessness during spaceflight [136]. Prolonged inactivity of skeletal muscles can also lead to an actual loss of muscle fibers [137–139], causing rarefaction to be underestimated when C/F is used to estimate capillarity.

Overoxygenation (hyperoxia) of muscle tissues is a likely cause of capillary rarefaction in sedentary muscles. Muscles use less oxygen when muscular activity decreases, which causes the muscles to be overperfused and hence overoxygenated. This overperfusion is expected to cause an autoregulatory vasoconstriction of muscle arterioles, which lowers blood flow to the muscle and thus decreases oxygen delivery. However, acute autoregulation of blood flow does not fully compensate for imbalances between oxygen demand and oxygen supply (i.e., the gain of the autoregulatory control system is not infinite), suggesting that some degree of hyperoxia should exist in muscles following a decrease in muscular activity, even in the face of arteriolar vasoconstriction. Direct measurements of muscle oxygen levels at multiple times following the initiation of muscle disuse will be required to confirm or refute the hyperoxia hypothesis.

3.4 LONG-TERM INCREASES IN BLOOD PRESSURE LEAD TO VASCULAR RAREFACTION

When the blood pressure is too high, excessive amounts of blood are literally pushed through the microcirculation. This overperfusion of existing microvessels leads to a loss of microvessels (microvascular rarefaction). Microvascular rarefaction is well-documented in the skeletal muscles of various rat models of hypertension. These models include spontaneously hypertensive rats [140–142], reduced renal mass-salt loading hypertension [143], and deoxycorticosterone-salt loading hypertension [144]. Also, hypertension caused by bilateral renal artery constriction can cause microvascular rarefaction in rat brain [145]. In hypertensive humans, microvascular rarefaction can occur in skeletal muscle [146,147], retina [148], conjunctiva [149], skin [150,151], and small intestine [152].

One explanation for these various findings in which high blood pressure leads to microvascular rarefaction is the following: High blood pressure increases blood flow to tissues beyond the level needed to maintain adequate oxygenation. The increase in tissue oxygenation leads to an autoregulatory vasoconstriction, which returns blood flow and tissue oxygenation to nearly normal levels. In this acute stage of autoregulation called *functional rarefaction*, some of the microvessels are not perfused with blood but are still present anatomically. Because the acute autoregulation of

blood flow cannot fully compensate for the excess in perfusion, the tissues continue to be overperfused and hence overoxygenated. Prolonged hyperoxia in the tissues leads to decreased levels of VEGF-A and other oxygen-sensitive proangiogenic growth factors. The result of long-term hyperoxia is a structural loss of microvessels (*structural rarefaction*). This hypothesis is supported by the following findings: (a) functional rarefaction precedes structural rarefaction in the skeletal muscles of spontaneously hypertensive rats [140], (b) basal levels of VEGF-A (protein and mRNA) are lower in skeletal muscle and heart of spontaneously hypertensive rats compared with normotensive Wistar-Kyoto control rats [153], (c) skeletal muscles of hypertensive humans have lower levels of VEGF-A protein (and lower capillary density) compared with normotensive control patients [154], and (d) capillary rarefaction in the skeletal muscles of spontaneously hypertensive rats was virtually eliminated by prolonged exposure to a hypoxic environment (the effect was abrogated by VEGF-A neutralizing antibodies) [153].

3.5 OXYGEN IS A MASTER SIGNAL IN GROWTH REGULATION OF THE VASCULAR SYSTEM

Why does tissue oxygenation dictate microvascular growth and regression? Many different metabolic fuels are required for cellular metabolism, but oxygen is especially critical because cells have limited stores compared with metabolic substrates such as glucose, fatty acids, and amino acids. This relative inability of tissues to store oxygen can explain why oxygen is a master signal in growth regulation and why oxygen falls to low levels in skeletal muscle within a few seconds following an increase in metabolic rate (Figure 3.4).

3.5.1 Increases in Muscular Activity Cause Decreases in Muscle Oxygenation

When the activity of a muscle increases, the muscle uses greater amounts of oxygen, which decreases the partial pressure of oxygen in the muscle. Figure 3.4 shows that contractions of the mouse gastrocnemius muscle causes the partial pressure of oxygen (pO_2) in the muscle to decrease rapidly to low levels.

3.5.2 Oxygen Regulates Angiogenic Growth Factor Production

Many proangiogenic factors and their receptors can be modulated either directly or indirectly by hypoxia or ischemia in poorly perfused tissues. These include but are not limited to the following: vascular endothelial growth factor (VEGF-A) [157–161] and its receptors, VEGFR1 [162–163] and VEGFR2 [164–166]; placental growth factor (PlGF) [167,168]; angiopoietin-1 (Ang1), angiopoietin-2 (Ang2) and their Tie2 receptor [158,169–174]; fibroblast growth factor-2 (FGF2) [175–179], and transforming growth factor-beta (TGFβ) [180,181]. In addition, oxygen regulates

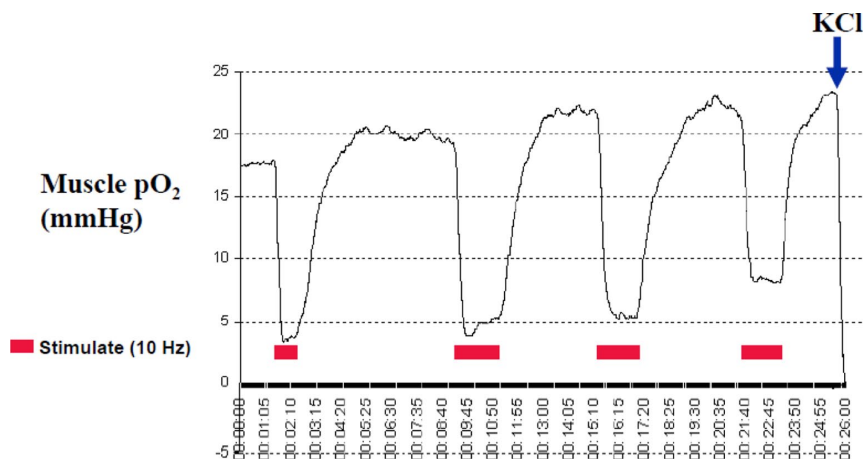


FIGURE 3.4: Increased muscular activity causes decreased oxygenation. A C57Bl/6 mouse was anesthetized with pentobarbital, skin was removed from the hind limb, and a channel was made parallel to the gastrocnemius muscle using a 27-gauge needle. An OxyLite® probe (160 μ m diameter) was placed into the medial head of the muscle by way of the needle channel. The first period of stimulation caused the muscle pO_2 to decrease to ~ 3 mm Hg. Similar values of pO_2 are found by magnetic resonance spectroscopy or near-infrared spectroscopy in exercising human skeletal muscle [75,155,156]. When stimulation was terminated, the muscle pO_2 increased to ~ 20 mm Hg, where it remained until the next period of stimulation. Note that successive bouts of stimulation caused the pO_2 to fall to a lesser extent and then to rise to a higher value when stimulation was terminated. This progressive rise in muscle pO_2 with repeated bouts of stimulation is consistent with the principles of blood flow autoregulation. Potassium chloride (KCl) administered via cardiac puncture stopped the heart, causing the muscle pO_2 to fall precipitously to a value of 0 mm Hg as shown, indicating that the probe had not drifted during the course of the experiment (T.H. Adair, unpublished observation).

expression of the transcriptional regulator, **hypoxia-inducible factor-1** (HIF1) [182–186], which in turn targets genes for VEGF-A [187–189], VEGFR1 but not VEGFR2 [163], and **inducible nitric oxide synthase** (iNOS) [190–192]. Also, a hypoxic environment can modulate antiangiogenic factors such as **endostatin** and **thrombospondin-1** (TSP1) [193–196].

3.5.3 VEGF-A Released From Hypoxic Tissues Is a Key Regulator of Angiogenesis

Shweiki et al. [197] discovered in 1992 that the proangiogenic factor, VEGF-A, is markedly up-regulated in glial tumor cells exposed to an anoxic environment. This finding was confirmed in vitro and in vivo in multiple laboratories [198,199] and was shown to be reversible when cells exposed to

physiological levels of hypoxia were returned to a normoxic environment [159]. Subsequent studies showed that cells deficient in oxygen produce the transcription factor, hypoxia inducible factor-1 (HIF1), which is a major stimulus for VEGF-A production in hypoxic tissues. HIF1 induction of VEGF-A expression occurs in skeletal muscle during exercise [75,200,201], ischemic heart [202], ischemic brain [203,204], ischemic retina [205], and hypoxic/ischemic tumors [206].

3.5.4 Negative Feedback Regulation of VEGF-A

Most control systems of the body act by a negative feedback mechanism [207]. In the case of angiogenesis, a low level of oxygen in the tissues causes the release of VEGF-A, which in turn stimulates angiogenesis. The development of new capillaries increases the supply of oxygen to the tissues, causing VEGF-A to return to nearly normal levels, thus closing the negative feedback loop. The possibility that VEGF-A could be subject to negative feedback regulation was first demonstrated in a rat model of endurance exercise training [208] (Figure 3.5). Note that VEGF-A mRNA increased to high levels after 4 days of muscle stimulation, and then decreased gradually over the next several days reaching low plateau values after 14 to 21 days of increased muscular activity. Capillary-to-muscle fiber ratio (C/F) increases during a 28-day period of muscle stimulation as shown in Figure 3.5 [209,210]; however, stimulating muscles for 60 days does not increase capillarity to a greater extent [211]. Thus, despite continued exercise, VEGF-A gene expression returned to nearly normal levels after 14–21 days of stimulation [208] (Figure 3.5). This temporal relation between muscle capillarity and VEGF-A expression can explain why VEGF-A production is thought to be regulated by a

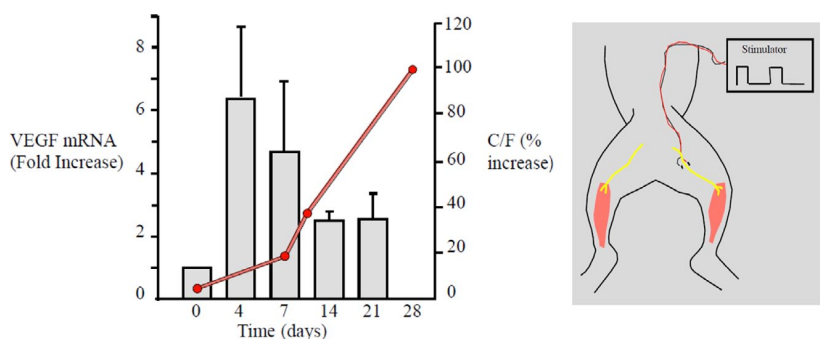


FIGURE 3.5: VEGF-A levels in rat anterior tibialis muscle during chronic electrical stimulation. The peroneal motor nerve of the one leg was stimulated for 2-h periods followed by 4-h periods of rest for 21 days. The contralateral anterior tibialis muscle served as a control. Stimulation parameters were 10-Hz, 300- μ s square wave pulses, and 3–5 V. VEGF-A mRNA (bars) was measured by Northern blot analysis. C/F, capillary-to-muscle fiber ratio (red line) [209,210]. Redrawn after Hang and Adair [208].

negative feedback mechanism. Presumably, the increase in capillarity induced by VEGF-A returns tissue oxygenation to normal, and VEGF-A expression, in turn, returns to nearly normal levels.

The possibility that VEGF-A is subject to negative feedback regulation has been substantiated in many laboratories. Chronic electrical stimulation of rabbit anterior tibialis muscles caused a 10-fold increase in VEGF-A mRNA expression after 4–6 days of stimulation and a return to control levels by day 14 [212]. Studies in treadmill-exercised rats show a sixfold increase in VEGF-A mRNA after 1–2 days of exercise, which decreased to control levels after ~12 days [213]. A study in humans [214] showed that a single bout of knee extension exercise caused a threefold greater increase in VEGF-A levels in untrained subjects compared with trained subjects where the muscle capillarity was already elevated. Feedback regulation of VEGF-A also seems to occur in rat brain exposed to a hypoxic environment [215]. Exposure to a hypoxic environment caused a maximum VEGF-A response (fivefold increase) in rat brain after 4 days of hypoxia, which was followed by a return of VEGF-A expression to basal levels by 7–21 days [215]. The increase in oxygen delivery afforded by increased capillarity apparently returned brain oxygenation to normal, thus causing VEGF-A expression to return to normal. Other studies provide evidence of VEGF-A feedback regulation in a mouse model of retinopathy [216]. Exposing mice to a hypoxic environment caused VEGF-A mRNA expression to increase greatly after 12 h of exposure and then to return to nearly normal levels over the next several days as the retinal vascularity increased [216].

3.5.5 Oxygen Plays a Central Role in Feedback Regulation of Vascular Growth and Regression

A simplified negative feedback control scheme that illustrates metabolic regulation of vascular growth and regression is shown in Figure 3.6. When oxygenation is inadequate, the tissues become hypoxic, and this hypoxic signal induces or suppresses various proangiogenic or antiangiogenic substances. Vascular growth follows. The increase in vascularity promotes oxygen delivery to the tissue cells by increasing both capillary surface area and maximum blood flow to the tissues, and decreasing diffusion distances between capillaries and **parenchymal cells**. When the tissues receive adequate amounts of oxygen even during periods of peak metabolic activity, proangiogenic and antiangiogenic factors return to nearly normal levels, and these negative signals, in turn, stop the further development of the vasculature, thereby closing the feedback loop [63,217]. The opposite is thought to occur when tissues are overoxygenated. Normal tissue growth, exercise, and hyperthyroidism can increase metabolic rate, whereas muscle disuse decreases metabolic rate. Vascular damage and exposure to a hypoxic atmosphere can lead to a primary decrease in tissue oxygenation, and exposure to a hyperoxic atmosphere, as well as muscle disuse, can theoretically lead to a primary increase in tissue oxygenation.

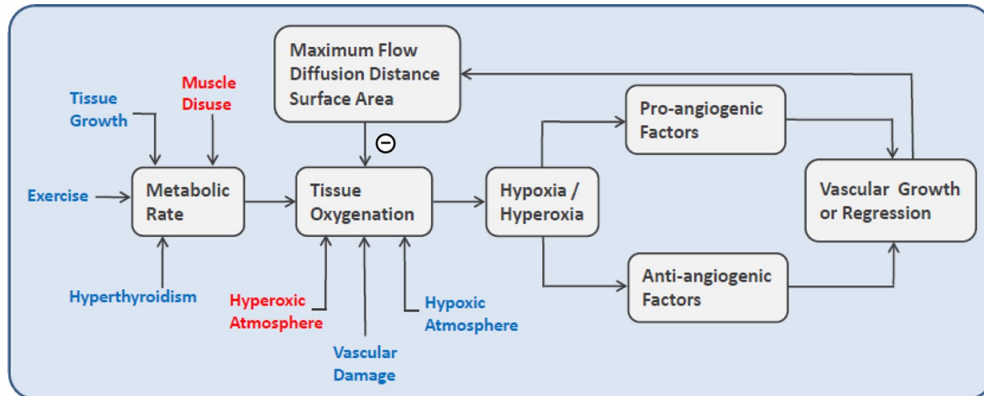


FIGURE 3.6: Central role of oxygen in metabolic regulation of vascular growth and regression. Factors listed in blue are thought to decrease tissue oxygenation causing hypoxia, which leads to vascular growth. Factors listed in red are thought to increase tissue oxygenation causing hyperoxia, which leads to vascular regression.

3.6 ROLE OF ADENOSINE IN METABOLIC REGULATION OF VASCULAR GROWTH

Adenosine is a nucleoside produced in all cells of the body by stepwise dephosphorylation of ATP. Hypoxic tissues produce adenosine from ATP, and the adenosine in turn functions to restore balance between oxygen demand and oxygen supply. Adenosine increases oxygen supply acutely by causing vasodilation and increased blood flow in the heart, skeletal muscle, brain, and other tissues [218–220]. Adenosine can decrease oxygen demand in the heart by multiple mechanisms [221–224]. For these reasons, adenosine is thought to serve as a negative feedback signal to maintain tissue oxygenation within a normal range.

Adenosine is also thought to have a long-term role in maintaining tissue oxygenation by stimulating angiogenesis [217]. Physiological concentrations of adenosine produced under hypoxic conditions stimulate a concentration-dependent proliferation and migration of endothelial cells obtained from both large and small blood vessels [225–232]. Endothelial cell proliferation and migration are key steps in the angiogenesis process necessary for establishing capillary sprouts in the microenvironment of a hypoxic tissue where adenosine levels are highest. Numerous studies have shown that adenosine or adenosine uptake inhibitors (which increase extracellular levels of adenosine) can stimulate blood vessel growth in various animal models [233,234]. The angiogenic actions of adenosine are thought to be mediated, as least partially, by its ability to increase the expression of VEGF-A: administration of adenosine [227,235–242] as well as the up-regulation of endogenous adenosine [228] can induce expression of VEGF-A in many cell types.

Figure 3.7 shows a mechanism for adenosine-induced angiogenesis under hypoxic conditions. AMP is dephosphorylated by ecto-5-nucleotidase, producing adenosine in the extracellular space adjacent to a parenchymal cell, which is the major source of adenosine under hypoxic/ischemic conditions. The parenchymal cell could be a cardiomyocyte, skeletal muscle fiber, hepatocyte, or any other functional element of an organ or tissue that is subjected to a hypoxic environment. Extracellular adenosine then stimulates the release of VEGF-A from the parenchymal cell by activating A_{2A} or A_{2B} receptors; adenosine activation of the A_1 receptor on macrophages can also stimulate VEGF-A production [243,244]. VEGF-A released from parenchymal cells and monocytes binds to its receptor (VEGFR2) on endothelial cells, stimulating proliferation and migration. VEGF-A is also a survival factor or maintenance factor for endothelial cells that may be regulated by adenosine under basal conditions [217]. Adenosine can stimulate endothelial cell proliferation independently of VEGF-A, which probably involves modulation of other proangiogenic and antiangiogenic growth factors or perhaps an intracellular mechanism [217]. In addition, hemodynamic factors associated with adenosine-induced vasodilation may have a role in development and remodeling of the capillaries as well as larger blood vessels. Once a new capillary network has been established and the diffusion/perfusion capabilities of the vasculature can supply the parenchymal cells with adequate amounts of oxygen, adenosine, and VEGF-A as well as other proangiogenic and antiangiogenic growth factors return to near-normal levels, thus closing the negative feedback loop [217].

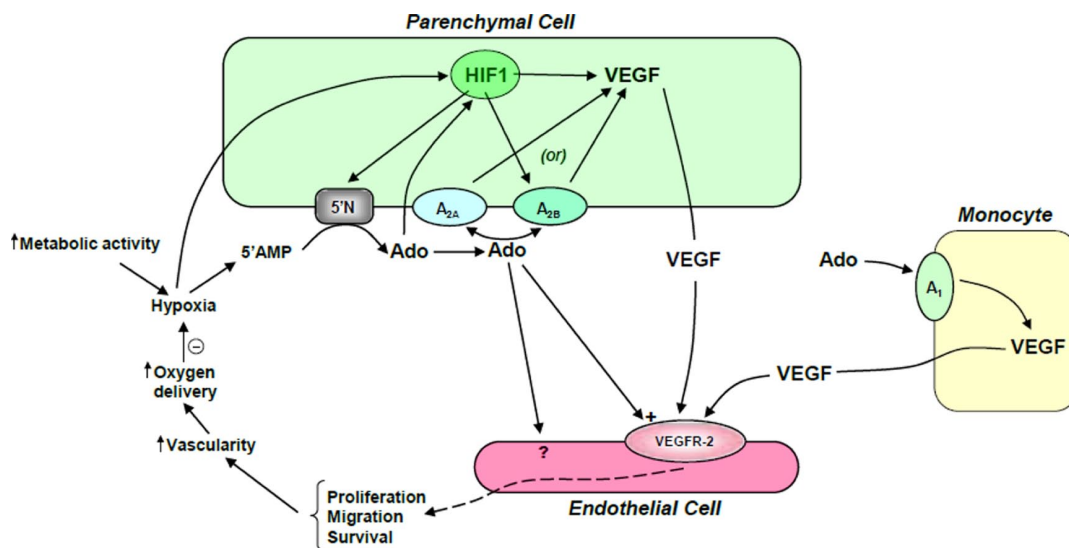


FIGURE 3.7: Mechanism of adenosine-induced angiogenesis. Ado, adenosine; HIF1, hypoxia inducible factor-1; VEGFR2, VEGF receptor-2; A_1 , A_{2A} , and A_{2B} , adenosine receptors; 5'N, ecto-5-nucleotidase; 5'AMP, 5'adenosine monophosphate. Redrawn and modified after Adair (2005) [217].

Multiple interactions can facilitate the adenosine induction of VEGF-A. Some of these interactions include the following: Adenosine can activate HIF1 by way of A_{2A} receptors in macrophages [245] and liver cells [246], which in turn can increase VEGF-A production. HIF1 can increase production of the adenosine A_{2B} receptor under hypoxic conditions [247]. The activity of ecto-5-nucleotidase (CD73) can be increased in the ischemic heart [248] and brain [249] of intact animals, possibly by way of a HIF1-dependent regulatory pathway [250]. Also, adenosine can increase the production of VEGFR2 in bovine retinal endothelial cells [251].

In addition, adenosine can modulate the expression of other angiogenic growth factors. An adenosine analog (NECA) increased the expression of the proangiogenic factors insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (FGF2) in human retinal endothelial cells [227]. NECA also increased the expression of the proangiogenic factors interleukin-8 (IL-8) and angiotensin-2 (Ang2) mRNA in human mast cells [237] as well as FGF2 in human microvascular endothelial cells [236]. Other studies have shown that adenosine and A_{2A} agonists can down-regulate the antiangiogenic factor tumor necrosis factor (TNF)- α in mouse macrophages [252]. TNF- α inhibits the proliferative response of endothelial cells through inactivation of VEGF receptors [253]. Therefore, adenosine can modulate multiple proangiogenic and antiangiogenic factors in a manner that promotes angiogenesis.

The available data indicate that adenosine might be an essential mediator for up to 50% to 70% of the hypoxia-induced angiogenesis in some situations [217]. This high estimate might be attributed to the fact that adenosine not only induces multiple proangiogenic growth factors (and inhibits release of antiangiogenic factors) but also incorporates mechanical hemodynamic factors through its vasodilatory actions that can promote development and remodeling of the vasculature.

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CHAPTER 4

Regulation: Mechanical Factors

Although the overall regulation of angiogenesis is dominated by metabolic factors in most tissues of the body, mechanical factors also play crucial roles in virtually every aspect of the angiogenic process. Migration of endothelial cells, tube formation (tubulogenesis), and pericyte/smooth muscle cell migration to newly formed endothelial sprouts are critical steps in the angiogenic process that depend upon mechanosensory mechanisms. These mechanosensory mechanisms need to be better understood because they represent control points in the angiogenic process that are not likely to be growth factor specific. In other words, regardless of the growth factor(s) that stimulate angiogenesis, the fundamental steps required to build new capillaries are essentially the same. A better understanding of the mechanosensory mechanisms could therefore provide the basis for unique therapeutic interventions to control angiogenesis.

4.1 CONTROL OF BLOOD VESSEL GROWTH

4.1.1 Epithelial Sodium Channel Protein Biology

One possible candidate for mediating mechanosensory events in angiogenesis is the epithelial sodium channel (ENaC), which is thought to form a mechanosensory complex. ENaC proteins are members of the degenerin (DEG)/ENaC protein family, sharing amino acid homology and protein structure [254–258]. Its members include non-voltage-gated sodium channels, neurotransmitter receptors, acid sensors, and mechanosensors. Two groups of DEG/ENaC proteins identified in mammals include ENaC and acid-sensing ion channels (ASIC). ASIC proteins are activated by protons: distribution has been reported in neural tissue and sensory epithelia [259–261]. ENaC proteins are distributed widely in multiple cell types in mammals. ENaCs play a rate-limiting role in epithelial Na^+ transport in kidney, lung, and colon [256–258,262–264]. They are comprised of at least four different protein subunits (α , β , γ , and δ), which are expressed in different combinations and with different subunit stoichiometries in a tissue-specific manner [257,262,263,265–267]. ENaCs are thought to require all subunits for full biologic activity; however, electrical current can be generated from channels composed of α -homomers and $\alpha\beta$ -, $\alpha\gamma$ -, and $\beta\gamma$ -heteromers [263,268–270]. ENaC proteins have been localized in vascular smooth muscle cells [271–278] and endothelial cells [271,279–282]: both cell types express α -, β -, and γ -subunit proteins [271–273,276,279].

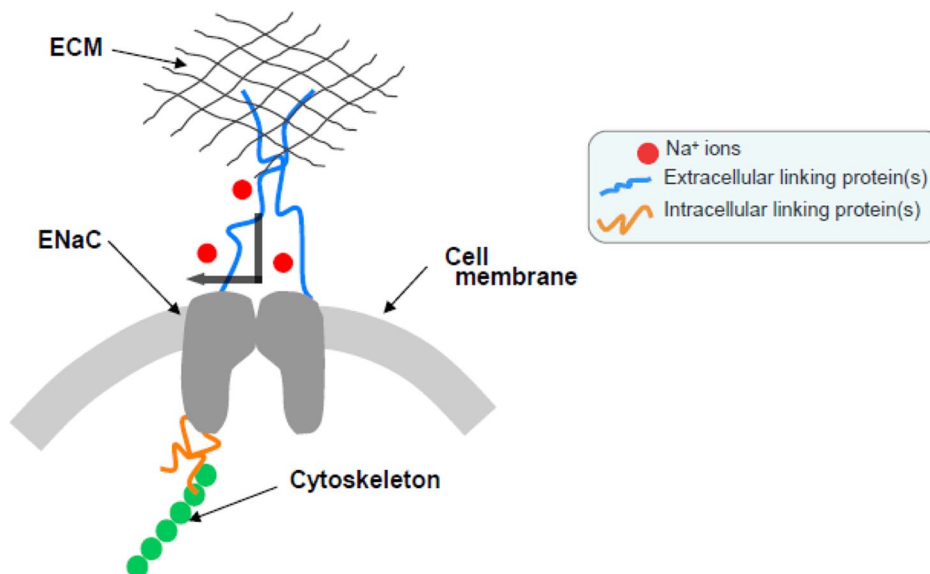


FIGURE 4.1: Model of mechanosensor with pore of epithelial sodium channel (ENaC) closed. ECM, extracellular matrix. Redrawn and modified after Drummond, Grifonia, and Jernigan (2008) [255].

4.1.2 Epithelial Sodium Channels Can Form a Mechanosensory Complex

A model of a DEG-dependent mechanosensor has been proposed to transduce mechanical forces to bioelectrical signals in nematodes. The model is based on genetic, biochemical, and functional analyses [256,257,262,264,283–285]. The mechanosensor (Figure 4.1) is thought to consist of an aqueous-filled protein channel that is tethered to the cytoskeleton and extracellular matrix (ECM), thereby allowing transmission of mechanical forces between the extracellular environment and the cell interior. The nematode mechanosensing/transducing complex may be fundamental to all metazoans, including mammals [262,285,286]. The main candidates for mechanosensors in mammals are members of the amiloride/benzamil-sensitive DEG/ENaC superfamily [254,261,287–289]: ENaC is speculated to form the aqueous-filled protein channel of the mechanosensing/transducing complex [262,285,286]. The structural nature of the channel tethers (i.e., linking proteins) is poorly understood; however, the COOH terminus of ENaC is physically and functionally linked to the cellular cytoskeleton through F-actin [290] and contributes to the control of channel activity by actin [291,292].

4.1.3 Epithelial Sodium Channels Can Mediate Mechanotransduction in Mammals

ENaC family members have been shown by immunocytochemistry to be expressed in mechanoreceptor structures in the rat foot pad [293], baroreceptors [294], sensory nerve endings in rat

larynx [295], sensory nerve endings of vibrissae [296], the muscle spindle [297], and vascular tissues [271–279,279–282]. Recent studies provide functional evidence that DEG/ENaCs play a role in physiological events that require mechanosensation/transduction. Shear stress can mechanically activate ENaC channels [289,298–300]. Amiloride/benzamil-sensitive ENaC channels contribute to mechanotransduction in mammalian muscle spindles [297]. Stretch-induced vasoconstriction (i.e., the myogenic response), the baroreceptor reflex, blood flow autoregulation, and migration of vascular smooth muscle cells can be attenuated using pharmacologic and/or genetic suppression of DEG/ENaC proteins [273–276,278,301–304].

4.1.4 Do Epithelial Sodium Channels Mediate Angiogenesis?

Recent studies suggest that ENaCs are required for angiogenesis [305,306]. In these studies, a specific ENaC inhibitor (benzamil) abolished both VEGF-A and FGF2 stimulated microvessel growth in the rat aortic ring angiogenesis assay [305,306]. The studies also showed that microvessel growth was reduced by about 50% in a mouse aortic ring angiogenesis assay with reduced levels of β ENaC (m/m), compared with aortas from normal littermates (+/+) [306]. In these angiogenesis assays, sprouting endothelial cells interact closely with fibroblasts, macrophages, and pericytes in an orderly sequence that recapitulates all stages of angiogenesis [53,54]. The microvessels are virtually indistinguishable from capillaries that form during angiogenesis in vivo and are composed of the same cell types [52,307–309]. Endothelial cells in the explant have not been modified by repeated passages in culture and they behave like normal endothelial cells in the intact animal [54]. These recent studies [305,306] therefore support the hypothesis that ENaCs play a critical role in the angiogenic process, possibly by acting as mechanosensors for migration of endothelial and vascular smooth muscle cells as well as endothelial tube formation.

4.1.5 Physical Forces Acting on the Walls of Blood Vessels

The walls of blood vessels are subjected to mechanical forces caused by blood flow, vasodilation, and blood pressure (Figure 4.2). Blood pressure causes a cyclical mechanical strain on the walls of arteries and arterioles (where blood pressure is pulsatile) and a constant strain in capillaries and veins where blood pressure is usually nonpulsatile. Because flowing blood exhibits a viscous effect, it tends to “stick” to the endothelium creating a **shear stress** that is proportional to the product of fluid viscosity and the velocity gradient between adjacent layers of the flowing blood [28]. Endothelial cells in all blood vessels are exposed to **shear stress**, which is a force that acts tangential to the endothelial cell surface causing morphological changes to endothelial cells (Figure 4.3). The walls of arteries and veins can also be stretched circumferentially as a result of vasodilation and compressed circumferentially as a result of vasoconstriction.

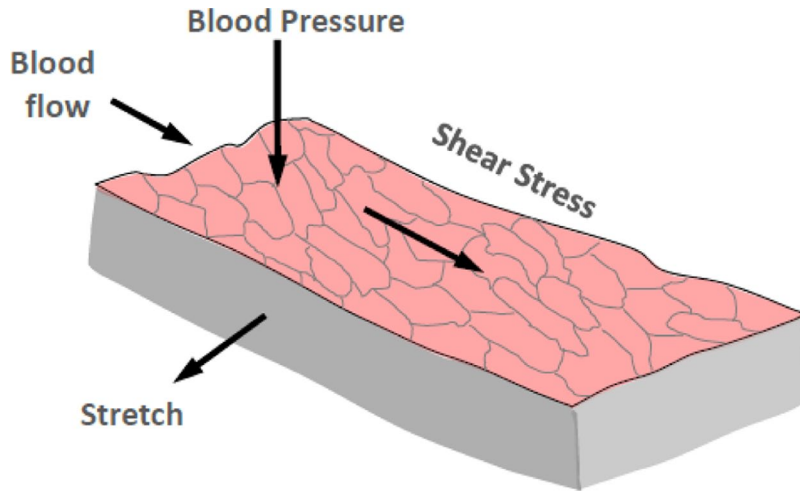


FIGURE 4.2: Physical forces caused by blood flow and blood pressure act on the walls of blood vessels. Flowing blood generates shear stress tangential to the endothelial cell surface. Circumferential stretch is caused by the action of blood pressure. Redrawn after Chien (2007) [28].

4.1.6 Shear Stress Is Sensed by the Endothelium

Molecular elements thought to play a role in sensing shear stress in endothelial cells are shown (Figure 4.4). These molecular elements include extracellular matrix (ECM), cell–ECM adhesion, cell–cell adhesion complexes, membrane components (ion channels, caveolae, surface receptors), and cytoskeletal filaments [311]. In addition, recent studies have suggested that epithelial sodium

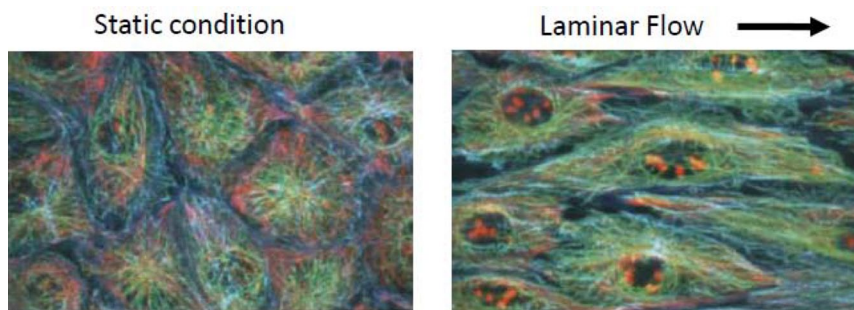


FIGURE 4.3: Effect of laminar flow on cytoskeletal organization and orientation of endothelial cells. Cytoskeletal elements are triple stained for actin (pseudocolor blue), microtubules (green), and intermediate filaments (red). Photomicrographs were taken under (left) static conditions and (right) 24 h after laminar shear flow at 12 dyn/cm^2 . Based on work by Galbraith et al. (1998) [310]. Used with permission from Chien (2007) [28].

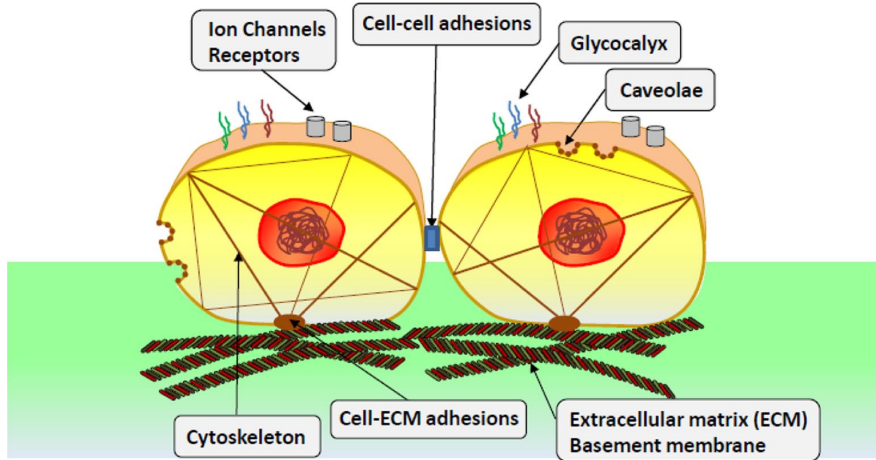


FIGURE 4.4: Elements of shear stress mechanosensing in endothelial cells. ECM, extracellular matrix. Redrawn after Balligand, Feron, and Dessy (2009) [311].

channels (ENaCs) may also play a role in sensing shear stress in multiple cell types [312–314]. Shear stress applied the luminal surface of endothelial cells is thought to be transmitted throughout the cell as well as to cell junctions and cellular adhesions to the ECM [311].

4.1.7 Increased Blood Flow (Shear Stress) Can Stimulate Angiogenesis

Thoma's [315] early observations in chick embryos that blood vessels with higher velocities of blood flow (higher shear stress) became larger whereas those with slower velocity atrophy have been substantiated in many laboratories in various animal preparations [316–320]. Mechanical factors associated with blood flow are thought to stimulate capillary development by intussusceptive angiogenesis [321,322]. In capillaries, intussusception refers to the splitting of single capillaries into two capillaries (Figure 4.5). Endothelial cells activated by shear stress [254] extend intraluminally forming two endothelial tubes through which blood can flow. Experimental proof for shear stress-induced angiogenesis has been achieved by chronic administration of vasodilators, primarily the α -adrenergic blocker, prazosin. Prolonged treatment with prazosin can increase muscle blood flow about threefold and stimulate angiogenesis [322,323–327]. Prazosin-induced angiogenesis could be VEGF-A-dependent [328]. Also, shear stress can activate the VEGFR2 pathway independent of VEGF-A [329]. Other vasodilators such as adenosine and dipyridamole (which increases adenosine levels in tissues) can also increase shear stress and stimulate angiogenesis; however, adenosine has multiple angiogenic actions independent of shear stress (Figure 3.7) [217]. Overall, the mechanism of shear stress-induced angiogenesis is poorly understood.

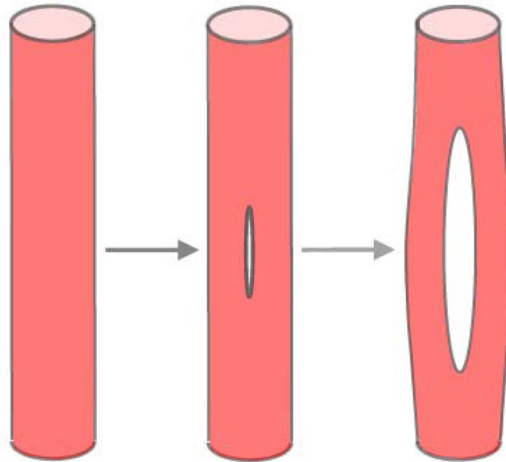


FIGURE 4.5: Shear stress-induced intussusceptive angiogenesis gives rise to longitudinal splitting of blood capillaries. Redrawn after Zhou et al. (1998) [322].

4.1.8 Possible Role of Endothelial Cell Shape in Regulating Blood Vessel Growth and Regression

The shapes of endothelial cells can dictate their rates of growth in vitro. Cells with different shapes are generated by treating plastic cultureware with surface-active agents that alter the adherence of cells [330]. Endothelial cells with a relatively flat configuration proliferate rapidly, whereas cells with a spheroid shape grow slowly [330]. Also, DNA synthesis increases in an exponential fashion in direct relation to linear increases in cell extension [331]. Electron microscopy studies [332] have shown that vasodilation literally pulls endothelial cells into the rapidly growing flat configuration, whereas vasoconstriction compresses endothelial cells causing them to become distorted with lower growth rates (Figure 4.6).

Does endothelial cell shape have a physiological role in growth regulation during long-term vasoconstriction and vasodilation? Long-term vasodilation occurs in tissues that have long-term increases in metabolic activity. The vessel growth that follows has been attributed mainly to proangiogenic growth factors released from hypoxic tissues. However, it is also possible that endothelial cell shape plays a role. Why? The flattened endothelial cells of dilated blood vessels are likely to be more susceptible to actions of angiogenic growth factors [330]. Long-term vasoconstriction can occur during the developmental stages of certain types of hypertension. Increased cardiac output leads to increased peripheral resistance (vasoconstriction) through an autoregulatory mechanism [333]. This vasoconstriction (functional rarefaction) is often followed by an actual loss of blood vessels

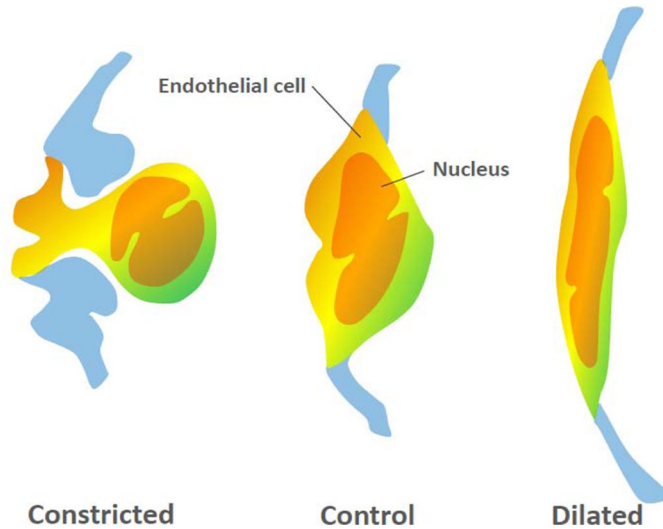


FIGURE 4.6: Model of endothelial cell shape during relative dilation and constriction of an arteriole. Redrawn after Stromberg et al. (1969) [332].

(structural rarefaction) [140,141]. Although the structural rarefaction might be explained by over-perfusion and hence overoxygenation of tissues with subsequent decreases in proangiogenic growth factors levels, it is also possible that endothelial cell shape plays a role. The compressed endothelial cells are likely to be less susceptible to the actions of proangiogenic growth factors, which would facilitate the rarefaction.

Although blood capillaries do not “vasodilate” directly, vasodilation of upstream arterioles increases capillary hydrostatic pressure and this can increase the capillary diameter and thus pull endothelial cells into a flat configuration. If this is true, and if the capillary endothelial cells release a stimulator of smooth muscle cell growth when they are pulled into a flat configuration, it could explain how capillaries that have a higher velocity of blood flow develop into larger vessels [316].

4.1.9 Mechanical Factors Have an Accessory Role in Angiogenesis

Neither blood flow nor mechanical factors associated with blood flow can actually regulate angiogenesis in heart, skeletal muscle, brain, and other tissues in which the vasculature has primary a nutritive function. Why? Because blood flow itself is regulated by metabolic factors in these tissues. For this reason, the proangiogenic actions of shear stress are thought to facilitate, but not regulate the angiogenesis. Likewise, those steps in the angiogenic process that require mechanosensation of physical stimuli serve to implement angiogenesis under the umbrella of metabolic regulation. There

are, however, instances in which flow itself can be considered a controlled variable in the negative feedback regulation of vascular growth. For example, lymphangiogenesis occurs when the rate of fluid loss from blood capillaries exceeds the fluid removal capacity of resident lymph vessels. It is also possible that the flow of interstitial fluid in the interstitial spaces of the kidneys plays a role in controlling angiogenesis in the peritubular capillary bed. This latter possibility will be addressed in future editions.

4.2 CONTROL OF LYMPHANGIOGENESIS

The concept that “**form follows function**” also applies to the lymphatic vascular system. The main physiological function of the lymphatic system is to pump extravasated fluid and proteins from the interstitial spaces back to the blood vascular system. Fluid and proteins leak continually from the blood capillaries into the surrounding interstitial spaces. The fluid enters into lymphatic capillaries and is pumped along a series of **lymphangions** all the way to the venous system where the lymph is returned to the blood.

The pulmonary lymphatic system can be overwhelmed with lymph when the left atrial pressure rises too high acutely. Normally, a rise in left atrial pressure to about 40 mm Hg causes an increase in lymph flow that is insufficient to remove interstitial edema fluid from the lungs (Fig-

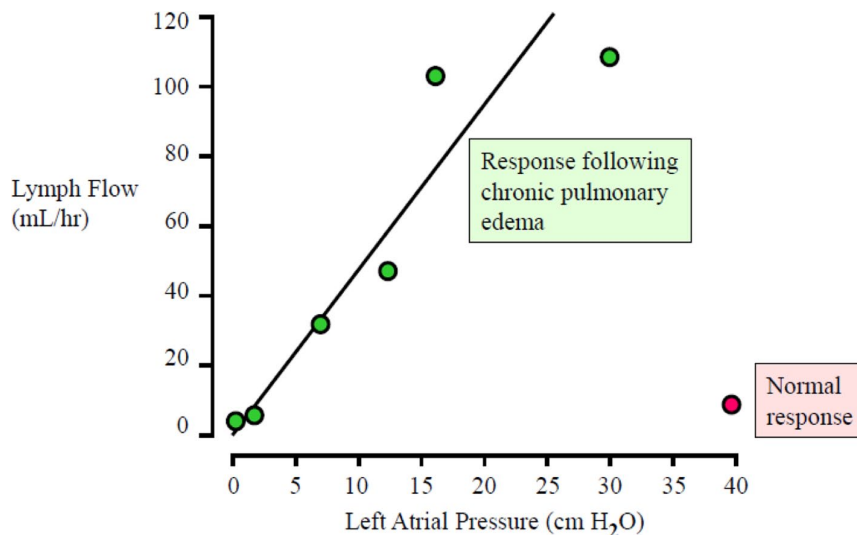


FIGURE 4.7: Lung lymph flow can increase to high levels in chronic pulmonary edema. When the lungs have excess amounts of interstitial fluid (edema) for long periods of time, lymphangiogenesis often follows. The growth of new lymph vessels or the enlargement of existing lymph vessels allows a given increase in left atrial pressure to cause a much greater increase in lymph flow compared with normal. Redrawn after Uhley (1962) [334].

ure 4.7). Pulmonary edema follows. However, with slowly developing chronic pulmonary edema, the pulmonary lymphatic system can adapt to the fluid challenge by growing new lymph vessels (lymphangiogenesis) and causing existing lymph vessels to grow larger. This growth of the lymphatic system greatly increases the amount of fluid that can be removed from the lungs, thereby helping to protect against pulmonary edema.

4.2.1 Flow-Guided Lymphangiogenesis

The possibility that lymphangiogenesis can be stimulated by a long-term fluid challenge is supported by studies in the mouse tail [335]. In these experiments, a circumferential section of skin containing lymph vessels was removed from the tail, but the underlying arteries and veins remained intact. The wounded area was then wrapped with a collagen matrix providing a pathway for interstitial fluid flow and a scaffold for cell proliferation and migration. Over the next several days, lymphatic endothelial cells were found to migrate along fluid channels in the collagen matrix; they eventually formed intact lymph vessels. These findings are in contrast to “blood angiogenesis” where fluid flow occurs only after a vascular channel has been established. The investigators also found increased expression of the lymphatic endothelial cell mitogen, VEGF-C [336] in the upstream regions of the collagen bridge [335]. In other studies using a similar model (Figure 4.8), VEGF-C therapy was shown to increase lymphatic endothelial cell density in the collagen matrix, and a primary inhibition of interstitial fluid flow was found to decrease lymphatic endothelial density [337]. Together, these findings support the hypothesis that growth of the lymph vessels can be regulated by the amount of interstitial fluid to which they are challenged.

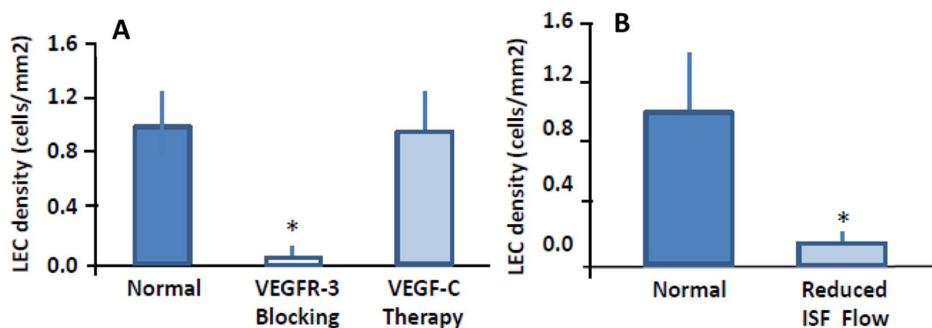


FIGURE 4.8: (A) Growth of lymphatic endothelial cells could be blocked with **VEGFR3** neutralizing antibodies and rescued with VEGF-C therapy. (B) An experimental reduction in interstitial fluid (ISF) flow led to a decrease in growth of lymphatic endothelial cells. Lymphatic endothelial cell (LEC) density was determined from immunostained cryosections of mouse tail skin. Redrawn after Goldman et al. (2007) [337].

4.2.2 High Salt Load Stimulates Lymphangiogenesis in Skin

Recent studies [338] in rats indicate that a high salt diet leads to sodium accumulation in the skin where the concentration can exceed 170 mmol/L. This high sodium concentration leads to increased density and hyperplasia of skin lymph vessels. The mechanism is thought to involve activation of tonicity-responsive enhancer binding protein (TonEBP) in macrophages that infiltrate the hypertonic environment of the skin interstitium. TonEBP binds the promoter of the gene that encodes VEGF-C increasing its secretion by the macrophages. These studies indicate that VEGF-C is an osmosensitive, hypertonically driven gene involved in lymphangiogenesis.

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Glossary

Angioblast also called *endothelial progenitor cell*, a mesenchymal cell derived from hemangioblast that gives rise to blood vessels.

Angiogenesis from the Greek word *Angēion*, meaning vessel, the formation of blood vessels from existing vasculature.

Arteriogenesis formation of arteries, which involves recruitment of smooth muscle cells into vessel wall.

Angiopoietin-1 a glycoprotein that activates its Tie2 receptor by inducing tyrosine phosphorylation. It promotes vessel maturation and stability.

Angiopoietin-2 an antagonist for the Tie2 receptor that counteracts the effects of angiopoietin-1.

Autoregulation (of blood flow) is a biological process in which tissue oxygenation is maintained within normal physiological limits by adjustments in arteriolar tone (acute autoregulation) and adjustments in vascularity/capillarity (chronic autoregulation). It is observed in heart, brain, skeletal muscle, and other tissues.

Blood islands clusters of angioblasts and hematopoietic precursor cells that give rise to different parts of the circulatory system. Fusion of blood islands along with lumina formation by angioblasts leads to primitive vascular network.

Chemokinesis an increase or decrease in the motile response of cells in random directions.

Chemotaxis the distance per unit time that a cell moves along a chemical gradient stimulus.

Coarctation (of the aorta) a congenital condition in which the aorta narrows in the area where it connects to the ductus arteriosus.

CD34 is a Cluster of Differentiation molecule (a glycoprotein) present on the cell surface that functions as a cell–cell adhesion factor. It is expressed in umbilical cord and bone marrow as hematopoietic cells, endothelial progenitor cells, blood vessel endothelial cells (but not lymphatic endothelial cells), mast cells, and other cell types.

Chorioallantoic membrane (CAM) is a highly vascular gas exchange membrane in bird eggs lying just beneath the shell surface that is composed of the fused chorion and wall of the allantois.

Delta-like 4 (Dll4) is a transmembrane ligand for Notch receptors that inhibits endothelial tip cell formation.

Disuse atrophy (of skeletal muscles) is muscle atrophy resulting from a lack of exercise due to a sedentary lifestyle, medical conditions that limit movement, or prolonged space flight at zero gravity.

Endostatin a 20-kDa C-terminal fragment of collagen type XVIII that has antiangiogenic properties.

Endothelial cell line luminal surfaces of cardiovascular system from heart to capillaries providing interface between blood and vessel wall. They are derived embryonically from angioblasts.

Endothelial progenitor cell a controversial cell type believed to circulate in blood and with the ability to differentiate into endothelial cells.

Extracellular matrix (ECM) fills spaces between cells and includes basement membranes. It is composed of fibrous proteins (e.g., collagen, elastin) and glycosaminoglycans. Provides support and anchorage for cells, serves as medium for diffusion exchange of nutrients and metabolites, and sequesters and releases various growth factors.

Fibroblast growth factor-2 (FGF2) also known as basic fibroblast growth factor (bFGF) is a member of the fibroblast growth factor family that is bound to basement membranes (BM) of blood vessels; its proangiogenic actions can be activated by heparin sulfate-degrading enzymes, which causes it to be released from BMs

Filopodia (singular, *filopodium*) thin cytoplasmic projections, similar to lamellipodia, which extend from migrating endothelial *tip cells* at the leading edge of a capillary sprout.

Folkman Judah Folkman (1933–2008) is considered by many to be the modern day father of angiogenesis, partly because of his pioneering studies showing that tumor growth is angiogenesis-dependent.



Form follows function (in biology) a fundamental law of nature in which the anatomical structure (form) of a system adapts to accommodate changes in the function of the system.

Gain of a system is the degree of effectiveness with which a control system maintains constant conditions. Very few systems have infinite gain because in most systems the correction (e.g., arteriolar dilation) is not sufficient to return the error (e.g., decreased oxygenation of tissues) all the way to normal.

Growth factors a natural occurring substance capable of promoting cellular growth.

Hemangioblast a multipotent cell and precursor to both hematopoietic stem cells and angioblasts.

Hematopoietic stem cell multipotent cell derived from hemangioblast that gives rise to all cell types in blood.

Hypoxia a deficiency in oxygen sufficient to enlist physiological mechanisms to correct the deficiency; occurs in normal and pathological states.

Hypoxia-inducible factor-1 (HIF-1) a transcription factor that responds to changes in oxygen levels in cells; HIF-1 levels increase during hypoxic conditions, and possibly decrease during hyperoxic conditions.

Inducible nitric oxide synthase (iNOS) NOSs are a family of enzymes that catalyze the production of nitric oxide from L-arginine.

Ischemia (Greek: *isch* = restriction; *hema* = blood) is a restriction of blood supply that can result in damage or dysfunction of the tissue. Ischemia is accompanied not only by hypoxia, but also a lack of glucose and other blood-borne metabolic fuels.

Krogh August Krogh (1884–1949) was a Danish physiologist who received the Nobel Prize in Physiology and Medicine for his many discoveries in cardiovascular physiology, especially those dealing with the microcirculation.



Lymphangiogenesis formation of lymph vessels from existing lymph vessels.

Lymphangion the functional unit of a lymph vessel needed to pump lymph. It is comprised of a short segment of lymph vessel delineated with proximal and distal one-way semilunar valves.

Mesoderm one of three primary germ cell layers lying between ectoderm and endoderm germ cell layers. It gives rise to multiple tissues including heart and large blood vessels.

Mesodermal stem cell gives rise to connective tissue, bone, cartilage, and the circulatory and lymphatic systems.

Microvascular rarefaction a reduction of microvessels.

Morphogen a hypothetical substance that controls cell position within a tissue and thus governs tissue development.

Multipotent progenitor cell gives rise to variety of oligopotent cellular progeny, which in turn give rise to limited assortment of terminally differentiated cell types. Unlike stem cells, they cannot reproduce themselves indefinitely.

Neovascularization a general term meaning formation of any blood vessel in adults.

Notch receptors a family of receptors (Notch1-4) with intracellular and extracellular domains present in all metazoans. Activation by ligands (such as Delta-like 4) leads to proteolytic cleavage and release of an intracellular domain, which enters the nucleus to alter gene expression. The notch signaling pathway is important for cell–cell communication.

Oligopotent progenitor cell gives rise to a limited assortment of terminally differentiated cells. Greek word *oligos* mean “a few.” Unlike stem cells, they have limited self-renewal capability.

Oxidative capacity is a measure of the maximal capacity of a tissue (usually muscle) to use oxygen; expressed as microliters of oxygen consumed per gram of tissue per hour.

Parenchymal cell the functional cell of an organ, e.g., skeletal muscle myocytes, cardiac myocytes, neurons, cells of nephrons, hepatocytes, etc.

Pericyte also called *mural cell*, attaches to capillary wall, providing support and maintaining quiescence of capillary endothelium. It can differentiate into smooth muscle cell, fibroblast, and other cell types.

Placental growth factor (PIGF) a member of the vascular endothelial growth factor (VEGF) subfamily secreted from the placental trophoblasts during pregnancy.

Progenitor cell gives rise to cells that continue to differentiate or to terminally differentiated cells. Unlike stem cells, they cannot reproduce themselves indefinitely.

Shear stress shear stress (in blood vessels) is a mechanical force caused by blood flow that acts tangentially to the endothelial cell surface of vessel walls. It is a function of blood velocity and viscosity.

Stalk cell a proliferating endothelial cell that elongates a sprout forming the trunk of a new capillary.

Stereology (Greek: *stereos* = solid) is a body of mathematical methods relating three-dimensional parameters defining the structure to two-dimensional measurements obtainable on sections of the structure.

Stem cell can undergo 'asymmetric division' producing daughter cell identical to parent cell as well as clonal progeny (*multipotent progenitor cells*) that continue to differentiate. They can accurately reproduce themselves indefinitely.

Tenotomy the cutting of a tendon either surgically (which can be therapeutic) or by trauma (which is not therapeutic).

Tetrodotoxin a neurotoxin that blocks action potentials in nerves by binding to the voltage-gated sodium channels of nerve membranes.

Thrombospondin-1 (TSP1) an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions; it has antiangiogenic properties.

Tie2 receptor an angiopoietin receptor; a tyrosine kinase that mediates cell signals by inducing phosphorylation of key tyrosines.

Tip cell a nonproliferating (or virtually nonproliferating) endothelial cell at the tip of a capillary sprout that does not have a lumen. They are characterized by filopodia extensions that direct migration.

Transforming growth factor-beta (TGF β) exists in three subtypes in humans (TGF β 1, TGF β 2, TGF β 3), can induce transformation of some cell types, and can play crucial roles in cell differentiation, embryonic development, regulation of immune system, and tissue regeneration.

Vasculogenesis (from Latin *vasculum*, meaning vessel) is the de novo formation of blood vessels from blood islands and angioblasts in embryos.

VE-cadherin VE (vascular endothelial)-cadherin also known as CD144 (CD, cluster of differentiation) is a glycoprotein required for maintaining a restrictive endothelial barrier.

VEGF-A vascular endothelial growth factor type A (also called VPF, vascular permeability factor) is a key proangiogenic growth factor.

VEGF-C Vascular endothelial growth factor type C induces selective hyperplasia of the lymphatic vasculature, i.e., causes lymphangiogenesis. Overexpression of VEGF-C in the skin of transgenic mice results in lymphatic (but not vascular) endothelial proliferation and vessel enlargement.

VEGFR1 vascular endothelial growth factor receptor-1 (also called flt1) is a receptor tyrosine kinase. It is thought to modulate VEGFR2 signaling possibly by acting as a decoy receptor since it has strong affinity for VEGF-A, but is weakly phosphorylated in endothelial cells.

VEGFR2 vascular endothelial growth factor receptor-2 (also called KDR/flk1) is a receptor tyrosine kinase, which mediates the angiogenic actions of VEGF-A as well as other cellular responses.

VEGFR3 vascular endothelial growth factor receptor-3 is a receptor tyrosine kinase that mediates lymphangiogenesis in response to VEGF-C and VEGF-D

Venogenesis the formation of veins; like arteriogenesis, it involves recruitment of smooth muscle cells into vessel wall.

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