Minireview

The diversity of endothelial cells: a challenge for therapeutic angiogenesis

Edward M Conway and Peter Carmeliet

Address: Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, University of Leuven, Gasthuisberg O&N, Herestraat 49, 3000 Leuven, Belgium.

Correspondence: Edward M Conway. E-mail: ed.conway@med.kuleuven.ac.be

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Abstract

Vascular endothelia comprise a diverse population of cells that specialize in response to genetic programs and environmental cues to take on distinct roles in different vessels, tissues, and organs, and in response to pathophysiological stresses. Characterization of endothelial-cell diversity will facilitate the development of novel, highly specific and safe therapies for many diseases.

The transition from an avascular embryo to a functional organism requires the formation of a highly complex network of capillary plexuses, blood vessels, and lymphatic vessels, in order to meet the demands of every organ. The formation of vessels in development and their later sprouting and remodeling are tightly regulated by an array of angiogenic and anti-angiogenic factors interacting with multiple cells and tissues [1,2]. Recent insights into these processes have led to a host of potential therapeutic strategies to directly treat diseases that are associated with too much angiogenesis (such as cancer or inflammation) or too little (such as myocardial or limb ischemia). Yet it is naive to assume that 'broad-spectrum' angiogenic or anti-angiogenic agents will be equally efficacious and safe for all diseases, each of which may tend to affect some vessels or organs more than others. Intuitively, it is reasonable to surmise that endothelial cells that line vessels in different organs have distinct functional and morphological features and thus will require tailor-made therapies. This presents a daunting challenge for the design of targeted approaches to prevent and/or treat disease.

Logically, the first step is to characterize the molecular and functional differences between endothelial cells in different kinds of blood vessel or vascular 'bed'. This is difficult, however, because the molecular steps that result in tissue-specific and organ-specific specialization of the vascular network are among those that are least well understood. Several groups have tried to delineate the molecular determinants that distinguish endothelial cells in different vessels and tissues, but the recent report by Chi *et al.* [3] is the first to use microarray techniques on a 'global' scale. In this study [3], the authors evaluated the expression profile of 53 cultured human endothelial cell lines from veins, arteries and microvessels from 14 different tissue sites. Not only did they readily discern patterns of gene expression that could distinguish between the endothelial cells of large and small vessels and between those of veins and arteries, but they also linked the expression patterns of gene products from specialized endothelia to functional roles that included regulation of lipid transport, immune-cell migration, neurogenesis, tracheal branching, and the establishment of the left/right asymmetry of the body. Overall, they provide definitive support for the notion that the transcriptional programs of endothelial cells from different tissues and organs are specifically adapted during development to assume distinct roles at each site.

Structural diversity of vascular endothelia

The concept of endothelial-cell heterogeneity in health and disease is not a new one. The architecture of the tumor vasculature was recognized in the early 20th century as being

distinct from that of normal tissue; tumor vasculature is characterized by irregularly shaped, dilated, and tortuous leaky vessels, often with associated hemorrhages. But how much endothelial-cell heterogeneity exists in normal growth and development? There is mounting evidence that endothelial cells are extraordinarily diverse in their morphology, function and gene-expression profile. Morphologically, they differ in size, shape, thickness, number of microvilli, and position of the nucleus. For example, aortic endothelial cells are generally thicker but cover a smaller area than those lining the pulmonary artery. Microvascular endothelial cells from human placenta at the end of gestation are elongated, whereas those from human umbilical veins are polygonal [4]; this distinction is reflected functionally by differences in their release of vasoactive substances and their interactions with leukocytes [5].

The most prominent structural differences that impact on function are in the apparent integrity of intercellular junctional contacts; these have been recognized by physiologists for many years. Small capillaries with tight, continuous junctions are most evident in the central nervous system, providing protection to the brain from bacterial or toxic insults. Somewhat thicker capillaries, also with continuous endothelium, are typically found in skeletal tissue and in the heart, testes and ovaries. Discontinuous endothelial cells, with gaps of variable size between cells (fenestrations), allow efficient transit of macromolecules and thus are predominant in endocrine glands and the kidney. The factors that determine the formation of tight junctions versus fenestrations are not well understood, although occludins, claudins, zona occludens proteins, and the family of junctional adhesion molecules are expressed in specific patterns in endothelial venules, blood-brain barrier endothelial cells, and lymphatic endothelial cells, contributing to the morphology and function of the intercellular contacts [6]. Recently, an endocrine-gland specific angiogenic growth factor (EG-VEGF) was isolated that is unrelated to the vascular endothelial growth factor (VEGF) family and acts via G protein-coupled receptors. EG-VEGF was found to cooperate with VEGF in the formation of capillary fenestrations [7], a discovery that supports the hypothesis that a variety of yet-to-be-identified growth factors regulate homotypic and heterotypic cell-cell contacts as well as specificity of endothelial-cell differentiation in different tissues.

Early studies showed that antigen expression is similarly diverse between endothelial cells in different organs and tissues [8,9], and this has been confirmed more recently. For example, the plasma glycoprotein von Willebrand factor is prominent in veins, less prominent in arteries, and largely absent from sinusoidal endothelial cells; the endothelial protein C receptor is predominantly expressed by large vessel endothelial cells [10]; and the cell-surface signaling proteins ephrin-B2 and ephrin-B4 are specifically expressed by arteries and veins, respectively [11]. The heart is particularly prone to cytokine-induced recruitment of damaging subsets of T lymphocytes; this may be partly attributed to higher expression of the cell adhesion molecule VCAM-1 in myocardial endothelial cells than in other organs [12].

Regulation of endothelial specialization

What are the mechanisms by which endothelial cells diversify, and when does this occur? Is the endothelial cell programmed, or are there environmental elements that regulate diversification and subsequent specificity? Environmental factors clearly affect organogenesis, and cross-talk between endothelial cells and the pericytes, stromal cells and extracellular matrix that surround them are essential for their coordinated function. This fact is highlighted by the finding that a phenotype like that of the blood-brain barrier can be induced in endothelial cells of non-brain origin by co-culturing them with astrocytes. This transformation, referred to as 'barriergenesis', is characterized by the formation of tight junctions between endothelial cells, triggered by the expression of several molecules, including Src-suppressed C-kinase substrate (SSeCKS), the platelet-derived growth factor PDGF-BB, the angiogenic and antipermeability factor angiopoieitin-1, its receptor Tie2, and the cell-adhesion molecule N-cadherin, by surrounding glial cells [13]. In the retina, an astrocyte 'template' is laid down prior to vascularization, and interaction between R-cadherin molecules on astrocytes and integrins and/or N-cadherins on endothelial cells or circulating endothelial progenitor cells (CEPs) mediates growth and migration of the vascular endothelium [14,15].

Although environmental cues appear to be critical for endothelial-cell specialization, genetic programming is equally important. It used to be generally believed that arteries and veins developed differently in response to differences in hemodynamic forces. Recent studies have revealed, however, that the distinction between artery and vein is determined during embryonic development, even before blood is circulating, and that Notch signaling is one of the crucial steps in determining the endothelial cell's phenotype. During vascular development, defects in signaling through the Notch pathway - which comprises ligands such as Jagged-1, Jagged-2, and Delta-like-4 and receptors such as Notch-1, Notch-2, and Notch-4 - disrupt normal differentiation into arteries or veins, resulting in loss of artery-specific markers such as *ephrin-B2* and ectopic expression in the aorta of venous markers such as *flt4* [16]. Conversely, overactivation of Notch suppresses differentiation of vessels to veins. Chi *et al*. [3] showed that Hey2, a transcription factor that is induced by Notch signaling, confers features of arterial endothelial-cell gene expression on vein-derived endothelial cells, upregulating arterial-specific genes, including *ADHA1*, *EVA1*, and *keratin-7*, while suppressing vein-specific genes, such as *GDF*, *lefty-1* and *lefty-2*. Fishman and colleagues [17] established in zebrafish that expression of the homolog of *Hey2*, *gridlock*, is required for

the early assignment of arterial endothelial identity, and defects in this pathway may be linked to morphogenic abnormalities of the aorta. These findings appear to refute the hypothesis that physiological cues are responsible for artery and vein differentiation. Several studies suggest, however, that even after endothelial cells attain a specific arterial or venous phenotype late in embryonic development, transdifferentiation may occur, and this process is regulated in part by the vessel wall [18]. Thus, a complex genetic program to regulate differentiation of endothelial cells into arteries and veins may be modulated by extrinsic factors, lending plasticity to the assembly and remodeling of the vascular network in health and disease.

This type of interaction between a genetic program and environmental factors may also hold for other kinds of endothelial differentiation, not just the choice between arteries and veins. Cells lining the endocardium and the coronary vessels are derived from progenitor cells migrating from distinct embryonic sites (reviewed in [19]). Fate-mapping studies indicate that diversification of these clonal cells takes place before their migration to the developing heart. Although this suggests that the fate of a coronary-artery endothelial cell is predetermined, the opportunities for the migrating cell to interact with other cells and factors are considerable, and thus diversification is likely to be a dynamic process, modified by intrinsic and extrinsic factors.

Studies of lung development have shown that when isolated lung rudiments with no blood vessels are implanted subcutaneously or underneath kidney capsules, they form lungs with vasculature that - remarkably - develops by both vasculogenesis and angiogenesis, with the characteristic vascular and alveolar network [20,21]. These findings support the concept that there are genetic programs for the development of the highly specific vasculatures but that these are modulated by extrinsic factors provided by surrounding cells, the extracellular matrix, and secreted growth factors and cytokines, thereby providing both plasticity and diversity.

The phenotypic plasticity and diversity of endothelial cells is not only manifest during embryonic development but is also central to the normal function of several organs. This is strikingly evident in the corpus luteum, a body that forms from an egg follicle after the egg is released. Morphological subtypes of microvascular endothelial cells in the corpus luteum have been defined according to their shape (epithelioid, spindle, round, or polygonal), the presence of cytoplasmic vacuoles, and the pattern of actin and vimentin filaments [22]. Distinct populations of these cells are more or less prominent at various stages of the monthly cycle of corpus luteum formation and regression. Formation of the corpus luteum includes a transient burst of angiogenesis, with growth and proliferation of endothelial cells that express high levels of cytokeratins, N-cadherin, and E-cadherin, and establishment of a continuous, tight-junctioned vascular network; this process is modulated by human chorionic gonadotropin, vasoactive peptides, and cytokines. With subsequent regression of the corpus luteum, the network is dissolved, as the endothelium transdifferentiates, yielding disrupted, discontinous intercellular junctions. Permeability increases, endothelial cells apoptose, capillaries regress and/or become occluded, and the corpus luteum degenerates, to prepare for the next cycle. Not only is this extraordinary endothelial-cell plasticity and diversity crucial for the normal luteal cycle, but endothelial cells from the corpus luteum of pregnancy also exhibit unique lectin-binding properties, distinct from those seen in the non-pregnant state.

The lymphatic circulation is composed of a network of thin-walled, discontinuous capillaries that carry fluid, macromolecules and immune cells. The extent of diversity of lymphatic endothelial cells has yet to be evaluated, but any knowledge of this may be important for our understanding of immune surveillance and how tumor cells metastasize through the lymphatic vessels [23]. Although the extrinsic and intrinsic factors that regulate the formation of lymphatic vessels and the specification of lymphatic organs and tissues are largely unknown, it appears that expression of the Prox1 transcription factor signals a switch in commitment from a venous endothelial phenotype to a lymphatic one [24]. Transcription-profiling studies of isolated cells have identified several markers that are notably upregulated in lymphatic endothelial cells compared with blood endothelial cells, including Prox1, LYVE-1 (a marker of unknown function), the chemokines CCL21 and RANTES, stromal cell-derived factor-1, and the angiogenesis regulator angiopoietin-2.

Angiogenic progenitors in therapeutic angiogenesis

Accumulating evidence suggests that angiogenic progenitor cells may be recruited from the bone marrow, circulation, and other tissues, and these may contribute to new vessel growth during normal development and after injury. In response to an angiogenic stress, circulating endothelial progenitor cells (CEPs) are mobilized, presumably from endothelial precursor cells (EPCs), which are predominantly located in the bone marrow. The CEPs proliferate and migrate to the site of injury under the guidance of a variety of factors, including VEGF, fibroblast growth factor 2, soluble Kit ligand, and insulin-like growth factor (reviewed in [25]). Although characterization of these progenitor cells is incomplete, it is clear that as the CEPs mature, their antigen expression pattern changes, with loss of the CD133 cell-surface marker (whose function is unknown) and acquisition of markers specific to the target tissue. Thus, for example, CEPs with a lymphatic-vessel fate upregulate expression of the VEGF receptor 3.

Major advances have been made in delineating the molecular mechanisms that regulate the processes that ultimately

lead to incorporation of EPCs and CEPs into newly forming vessels. Indeed, preclinical trials have been initiated to use transplantation of angiogenic progenitor cells to enhance tissue revascularization of damaged heart, limb and retina. Yet potential hurdles remain. The degree to which CEPs can adapt to different vascular beds, in a variety of pathological states, is entirely unknown [26]. The heterogeneity of the vasculature and the complex regulation of specialization of the endothelium may hinder effective incorporation of transplanted CEPs. For example, injured tissue may fail to provide appropriate organ-specific or tissue-specific environmental cues to ensure transdifferentiation of CEPs into functionally mature and competent endothelial cells. In the worst case, this may yield abnormal, non-functional, bleeding or thrombosis-prone vessels in undesirable locations, and furthermore it may exacerbate disease at other sites. Nonetheless, vascular endothelial progenitor-cell therapies hold great promise. Characterization of the factors that regulate the differentiation and specialization of EPCs, CEPs, and mature endothelium will facilitate safe introduction of this novel and exciting technology to the clinic.

With wide acceptance that the vascular endothelium is composed of cells with considerable heterogeneity, several groups have devised novel methods to identify tissue-specific receptors that might be used therapeutically. Differential molecular phenotyping of endothelial cells from normal and tumor tissue using serial analysis of gene expression (SAGE) technology has revealed the expression of novel tumor-specific endothelial markers [27]. Differential-display technologies have been used to distinguish the gene profiles of endothelial cells under shear stress, demonstrating that this stress induces expression of protective anti-oxidant, anti-apoptotic and anti-proliferative genes [28]. Using phage-display of random peptide libraries, peptide ligands have been isolated that recognize endothelial cells from specific tissues or organs, including the breast, lung, prostate, and heart, and this technique may be expanded to map the expression profile of the vasculature under different stresses [29]. The results of such studies have been exploited by linking pro-apoptotic factors to the peptides so as to selectively destroy murine prostate tumors [30]. In experimental models, other agents, including neutralizing antibodies and cytotoxic drugs, have similarly been targeted to vascular endothelial cells in specific tissues to block tumor angiogenesis, interfere with retinal neovascularization, and to suppress the chronic inflammation associated with arthritis (reviewed in [29]).

The complexity and heterogeneity of the vascular endothelium, once viewed as an inert conduit to simply carry blood, is astounding. With enhanced computer database management, the development of high-throughput *in vivo* screening methods, and paradigms to explore the diversity of endothelial cells, smooth muscle cells and extracellular matrix under a variety of pathophysiological conditions using *in vivo* experimental models, the stage is set for the development of safer, more effective and targeted therapies for the many diseases associated with vascular dysfunction.

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