Mechanisms of Endothelial Differentiation in Embryonic Vasculogenesis

J.E. Ferguson III, Rusty W. Kelley, Cam Patterson

Abstract—The formation of new blood vessels in the adult organism not only contributes to the progression of diseases such as cancer and diabetic retinopathy but also can be promoted in therapeutic approaches to various ischemic pathologies. Because many of the signals important to blood vessel development during embryogenesis are recapitulated during adult blood vessel formation, much work has been performed to better-understand the molecular control of endothelial differentiation in the developing embryo. In this review, we describe the current understanding of where endothelial differentiation from pluripotent progenitor cells occurs during development, how this process is controlled at the molecular level, and what model systems can be used to investigate the earliest steps of blood vessel formation. (Arterioscler Thromb Vasc Biol. 2005;25:2246-2254.)

Key Words: development ■ endothelium ■ vasculogenesis ■ embryogenesis

Consistent with its central importance in embryonic development, the history of research into vascular development is populated with seminal histologists such as Wilhelm His and Florence Sabin.1 Ideas about endothelial origin followed soon after the development of histology as a technique and cell biology as a field of study. Beyond characterization of the fundamental anatomy of vascular development (in many timeless analyses that bear rediscovery by present-day investigators), the first investigators in this field participated in one of the classic debates in all of developmental biology: where and when do endothelial cells (and hence blood vessels) arise in the developing embryo? Because blood vessels are first observed in the yolk sac in avian and mammalian embryos, it was initially assumed that all blood vessels arise from extraembryonic tissues. However, careful histological analysis subsequently indicated that isolated foci of endothelial cells can also be observed in the embryo proper, which suggested that blood vessels arise from an intraembryonic source (specifically, the mesoderm) rather than via colonization. The latter model has been successively confirmed by increasingly sophisticated approaches to isolate and track blood vessel development in mammalian systems (discussed below).

Gastrulation and Post-Gastrulation Events

Although there has been considerable debate about what is a surprisingly elusive question, it is now generally accepted that the mesoderm is the exclusive source of endothelial cell progenitors. Although efforts have been made to identify specific loci within the mesoderm that preferentially specify endothelial fates, it now seems clear that precursors to endothelium arise within all intraembryonic mesoderm, with the exception of the notochord and prechordal plate, as loose aggregations of cells bearing early markers of the endothelial lineage with the potential to coalesce into vascular cords.2

The signals for induction of angioblasts (defined by Gray’s Anatomy as “individual vasoformative cells”) are not entirely clear. Initiation of vascular differentiation within the embryo occurs in opposition to endoderm, which suggests that endoderm-derived signals are required for angioblast commitment within mesoderm.3 Several signals are appropriately expressed in a spatial and temporal pattern within the
endoderm to fulfill this role and are required for normal vascular development. However, recent evidence suggests that angioblast commitment is not totally dependent on endoderm-derived signals, although later morphogenetic steps in vascular development may require endoderm–mesoderm interactions.

The pluripotency of vascular precursors has also been a vexing issue within the field. The close colocalization of endothelial and hematopoietic precursors (so-called blood islands) within the yolk-sac has been recognized since early in the 20th century. This raised the possibility that both lineages arise from a common precursor, the hemangioblast. Subsequent studies have indicated that both lineages bear certain common molecular markers, and cells with the potential to produce either lineage have been isolated from differentiating mouse embryo bodies. The pluripotency of these putative hemangioblasts has not been completely characterized, although some data support the possibility that these multipotential cells may also assume characteristics of the smooth muscle lineage under appropriate inductive conditions. It also does not seem likely that hemangioblast identity is a required step for all endothelial development, and in fact direct differentiation of angioblasts from mesoderm is also a well-supported phenomenon.

Despite data supporting the existence of the hemangioblast, definitive isolation of these cells and localization within the developing embryo has been an extraordinary challenge. However, recent evidence indicates that cells with hemangioblast properties are present transiently in the posterior segment of the primitive streak during gastrulation and are defined by the coexpression of the vascular endothelial growth factor receptor flk1 and the mesodermal T-box gene brachyury. These data also suggest that endothelial and hematopoietic fates of embryonic cells are established before the appearance of yolk-sac blood islands during development. In fact, some data suggest that the hemangioblast lineage may be committed before gastrulation within the epiblast. Despite this controversy, and for the sake of clarity, the term “hemangioblast” will be used throughout the remainder of this review to describe bipotential endothelial cell precursors.

Extraembryonic Vasculogenesis
Blood vessel formation is classically divided into 2 categories. Vasculogenesis refers to the in situ differentiation of endothelial cells to form blood vessels, with or without associated angioblast migration. In contrast, angiogenesis refers to the formation of new blood vessels via extension or remodeling of existing blood vessels. Angiogenesis occurs throughout development and in adulthood, whereas vasculogenesis is generally thought to occur during a limited period early in embryonic development. (The term “vasculogenesis” is occasionally used to refer to the development of blood vessels during adulthood, especially when associated with circulating vascular progenitor cells. However, given the distinct developmental mechanisms of each process, this seems to be an inappropriate use of this term based on its natural definition.) Vasculogenesis is further subdivided based on whether it occurs within the extraembryonic or intraembryonic compartments. The best available evidence suggests that these 2 waves of vasculogenesis are temporally and spatially distinct, and molecular studies indicate that they are also partially distinct at the mechanistic level.

Extraembryonic vasculogenesis precedes intraembryonic vascular development, and in mammals is first apparent as blood islands assembling within the mesodermal layer of the yolk sac (Figure). Blood islands are foci of hemangioblasts that differentiate in situ, forming a loose inner mass of embryonic hematopoietic precursors and an outer luminal layer of angioblasts. Blood islands eventually coalesce into a functional vascular network that constitutes the vitelline circulation, which is adapted to transfer nutrients from the yolk sac to the embryo proper. Recent evidence indicates that extraembryonic blood vessels may also arise independently of blood islands via direct differentiation of angioblasts from mesoderm. Vessels arising via yolk sac vasculogenesis communicate with the fetal circulation via the vitelline vein, but otherwise do not contribute to intraembryonic vasculature.

De novo extraembryonic vasculogenesis also occurs in the allantois, a structure responsible for the induction of placental development and for the formation of the umbilical vessels. Avascular allantoides can be excised from the developing embryo and cultured in isolation for ~1 day, during which time they develop a definitive vascular plexus. This indicates that vasculogenesis can proceed independently in the allantois during development. Because of this, primitive vessels are already in place in the allantois as it makes contact.
with the chorion to facilitate the formation of the maternal-fetal circulation. Whether these cells also contribute to hematopoiesis and whether they receive inductive signals from endoderm or allantoic mesothelium is still undergoing debate, and may differ between species.18–20 Furthermore, given the close proximity of the developing allantois to the yolk sac mesoderm and posterior primitive streak, 2 structures with potent hemangioblastic potential, further studies have sought to address the exact temporal and anatomic origins of the vasculogenic cells resident in the allantois.21

**Intraembryonic Vasculogenesis**

Although recent evidence suggests that intraembryonic vasculogenesis can occur throughout most of the intraembryonic mesoderm, much work has been performed to characterize the different origins of particular vascular structures. As might be expected, the endocardium and great vessels are the first intraembryonic endothelial structures formed during development.9 The endocardium originates in mammals from clusters of migrating angioblasts derived from preomitic cranial mesoderm that enter the pericardial area to form a vascular plexus adjacent to the developing myocardium. This plexus undergoes remodeling to form the endocardial tube, which is the first vascular structure formed in the mammalian embryo.9 Simultaneous to heart development, vasculogenesis is initiated within the aortic primordia, a collection of mesoderm just lateral to the midline, to give rise to the dorsal aortae and the cardinal veins.22 During this development, differentiating angioblasts assemble into primary vascular networks, which are then remodeled in a bidirectional fashion to generate the bilateral embryonic aorta.9 This region, later termed the para-aortic splanchnopleure (PAS), and then the aorta-gonad-mesonephros (AGM) region, continues to be a “hot-spot” for hematovascular differentiation during development (Figure).12

As the heart enlarges, passive diffusion of nutrients and waste becomes limiting, and a coronary vasculature is formed to supply the metabolically active heart tissue. Vascular precursor cells (including endothelial and smooth muscle cell progenitors) reside in the pro-epicardium (PE)—a mass of cells that appears to originate from splanchnic mesoderm in conjunction with the septum transversum just inferior to the heart.23 This cell mass makes contact with the developing heart tube and quickly spreads over the entire heart. Once spread, these cells undergo epithelial-to-mesenchymal transformation (EMT), and invade the underlying mesoderm, where they then give rise to the capillaries, veins, and arteries of the coronary vasculature.24 The development of the coronary circulation is clearly unique, and still requires investigation to determine the timing and molecular nature of the signals necessary to induce endothelial differentiation in the PE.25 26

**Molecular Regulation**

The anatomic basis for vascular development in the embryo has been clarified through studies that have been performed over the past century. In contrast, the molecular underpinnings for endothelial cell differentiation have only become clear in the last decade. In addition to cell-specific genetic determinants of endothelial differentiation, soluble factors are released that influence the fate of multiple cell lineages. Gain- and loss-of-function studies have helped us identify some of the participants in vasculogenesis. Specifically, targeted gene inactivation studies have better-defined the molecular regulatory mechanisms underlying these processes (Table). In this section, we review briefly the inter-relationships among some of the identified steps important to endothelial differentiation.

**Initiation**

Key to any inductive process in the embryo is the elaboration of signals that are integrated to mark the initiation of a developmental cascade. In the case of early events in endothelial differentiation, these cues must determine the spatial and temporal appearance of hemangioblasts from undifferentiated mesoderm, and also the further maturation and assembly of these precursors into nascent blood vessels. Of the well-defined signaling pathways, the best data indicate that crucial roles exist for members of the fibroblast growth factor families as proximal inductive cues for the hematovascular lineage. FGF appear to be key components required for hematovascular differentiation in differentiating embryonic stem cells.27 Concurrent with FGF signaling, BMPs, specifically BMPs 2 and 4, signal through downstream Smad proteins to help modulate early vascular development as indicated by gene deletion experiments.28–31 The potent effect of the endogenous endothelial BMP inhibitor BMPER in suppressing endothelial differentiation also highlights the importance of this signaling system in vasculogenesis.32 In addition, Indian hedgehog is implicated as an endoderm-derived signal for vascular competence.4 Definitive endoderm and mesoderm specification is also thought to be mediated in part by Brachyury or T, a transcriptional target of the Wnt signaling pathway.33 Wnt signaling pathways are essential during gastrulation, specifying pattern formation, and regulating stem cell differentiation during mammalian development.34–36 However, remarkably little is known about how these upstream factors interact to specify vascular identity of precursors. In addition, none of these signals has specificity for the vascular lineage.

The first secreted molecule with specificity for the endoderm during development is vascular endothelial growth factor (VEGF), and the role of VEGF family members in developmental and adult angiogenesis is now well-described. Mice lacking a single copy of the VEGF gene die early in development, indicating the critical role for this signaling pathway in vascular development.37 Cells that respond to VEGF must first express its receptors, Flk1 and Flt1 (VEGFR2 and VEGFR1). This indicates that VEGF itself cannot be the most proximal signal for endothelial differentiation, and it is still not clear precisely when VEGF first participates in vascular specification. VEGF receptors were thought to be restricted to the endothelial and early hematopoietic lineages; however, Flk1+ mesodermal progenitor cells were recently reported to contribute to muscle lineages,38 although these data have not been confirmed by other investigators. VEGF also binds the semaphorin receptor, neuropilin-1 (NP1), with high affinity in a complex that
reportedly enhances VEGF binding to Flk1. Both of the neuropilin coreceptors (NP1 and NP2) are necessary for proper vessel and yolk sac development. Flk1 also forms a complex with VE-cadherin, β-catenin, and PI3-K to intercede a VEGF-A survival signal through activation of the downstream messenger AKT, followed by a decrease in Bcl2-mediated apoptosis. Interestingly, in addition to playing roles in endothelial anchoring and VEGF-mediated survival, the signaling molecule β-catenin also participates in the canonical Wnt pathway during embryogenesis. Additionally, VEGF-A is expressed as multiple splice variants with varying abilities to bind heparin sulfate, producing freely diffusible VEGF-A isoforms as well as those that are retained in the extracellular matrix. Signaling of multiple VEGF splice variants among multiple receptors and coreceptors makes it difficult to correlate cellular and molecular function in nonlinear cross-talking pathways.

**VEGF/Flk1 Regulation**

Several upstream factors have been shown to regulate VEGF and Flk1 expression. The transcription factor hypoxia-inducible factor 1 (HIF-1) and its family members play crucial roles in sensing changes in tissue oxygen tension and stimulating gene expression changes that enhance blood vessel growth into hypoxic tissues during post-gastrulation development. Additional factors such as specificity protein 1 act in concert to regulate VEGF transcription. Evidence supports BMP signaling as a proximal stimulus for Flk1 expression.
and at the transcriptional level there appears to be necessary roles for GATA family proteins as well as the homeodomain protein HoxB5 in upregulation of Flk1 during development.46 Another layer of regulation in endothelial development is ascribed to the ETS family of transcriptional factors that direct downstream endothelial specific expression of Flk1, Flt1, the angiopoietin receptors, and MEF2C, a recently identified member of the MADS box superfamily of vascular developmental transcription factors.47 A genetic mutation of an unknown gene, termed “cloche,” leads to Flk1 deficiency with a subsequent failure in vasculogenesis.58,49 Although still incompletely characterized, upstream cues that initiate VEGF and Flk1 expression, and other regulators of endothelial differentiation, are likely participants in early specification of the vascular lineage.

Functional Markers of Endothelial Differentiation

Identified markers of endothelial cells are used to track their transition from the early stages of stem cell differentiation to the mature vessel, as well to distinguish them from other lineages, such as hematopoietic and smooth muscle. Not surprisingly, many of the molecules used to track endothelial identity are functionally important. The appearance of Flk1 expression is at the present time the earliest marker available for the endothelial lineage during development.8,50,51 Flk1 is expressed early in endothelial and hematopoietic cells, and persists in mature endothelium but not mature hematopoietic lineages. This pattern suggests that Flk1 may be a marker for hemangioblasts during early development, and this is born out by studies that indicate that the coexpression of Flk1 with mesoderm-derived transcription factors Brachyury or Scl/Tal1 denotes hemangioblasts.9,15 It is also important to note that because Flk1 is expressed by angioblasts as well as mature endothelial cells, it cannot be used solely to distinguish between different stages of endothelial cell differentiation. As the endothelial lineage progresses, the expression of brachyury followed by Scl/Tal1 are lost, whereas the Flk1 marker is retained.52 Elimination of Scl/Tal1 expression arrests hematopoiesis, but allows for endothelial cell progression.52,53 Scl/Tal1 and FGFR-1 are suggested to be linked in the segregation of hemangioblasts into hematopoietic and endothelial lineages.52 The entire transcriptional program of the endothelial lineage is yet to be established, and this represents a major limitation in our understanding of how cell-autonomous mechanisms coordinate development of vascular cell lineages.

Combinations of endothelial specific markers including VE-cadherin, PECAM-1, Tie-1, Tie-2, Flk1, and Flt1 are commonly used to trace endothelial differentiation.54 As might be expected, these markers also serve in functions vital to endothelial formation, remodeling, and maintenance. For example, VE-cadherin is expressed from the committed angioblast to the mature endothelial cell, but not in hematopoietic progenitor cells. VE-cadherin, an adhesive cell–cell recognition protein, participates in cell-sorting during vascular morphogenesis. With the ability to anchor to the cortical actin cytoskeleton via catenin proteins and vinculin, VE-cadherin offers junctional strength between endothelial cells.41 Mice deficient for VE-cadherin fail to couple a VEGF-A/Flk1–dependent endothelial survival signal to β-catenin and PI3-K, resulting in early embryonic death caused by severe vascular defects.41,54 PECAM-1 and Tie also function during endothelial differentiation with roles in adhesion and vascular network formation, respectively.55 In fact, PECAM-1 is commonly used to trace endothelial morphological development (ie, sprouting vessels).46,56 Although the fine points of their signal transduction pathways remain poorly understood, the function of markers used to identify endothelial cells and their progenitors have provided us a framework for reaching a more complete understanding of vasculogenesis.

Additional Regulatory Cues

Downstream of the early events, the cues required for blood vessels to assemble become better known, if also more complex. Among paracrine factors, the angiopoietin family members bind to the tyrosine kinase receptors Tie-1 and Tie-2, which are structurally similar to the VEGF receptors and play crucial roles in endothelial cell survival and remodeling of capillary plexi.57 Platelet-derived growth factors are required for recruitment of pericytes and smooth muscle cells to invest developing arteries and establish vasomotor tone.58 Several transcription factors are known to participate in vascular assembly including vascular endothelial zinc finger,59 GATA proteins,60 several members of the Krüppel-like family of zinc finger proteins,61 and Ets proteins,62,63 which have all been linked to steps in endothelial cell differentiation by virtue of their cellular phenotypes of defined transcriptional targets. Homeodomain proteins such as HoxB3 and HoxD3 participate in morphogenetic events responsible for vascular tube formation.64 Egfl7, a recently identified secreted factor in endothelial cells, is crucial for the separation and arrangement of angioblasts followed by endothelial cell assembly into cord-like vessels.65 Concomitant and subsequent to endothelial cell convergence is the networking of blood vessels. Molecules such as neuropilins and plexins, the 2 classes of semaphorin receptors, are known to direct microvessel branching and/or guidance to their target sites.60,66 An obvious conclusion from this cornucopia of studies is that blood vessel formation requires multiple pathways that talk among one another to coordinate the spatial and temporal differential gene expression pattern during blood vessel formation.

Vessel Fate

Interestingly, the fate of arterial, venous and lymphatic vessels are in part genetically determined early during vascular development before circulation and during the amalgamation of blood islands in the yolk sac. Molecules such as ephrins, ephrin receptors, neuropilin receptors, and type-A plexins serve as markers for arterial and venous identity, and appear well before the structuring of tube-like vessels.67 Arterial cell fate appears to be determined by intersecting signaling cascades involving first sonic hedgehog (Shh), then VEGF, followed by the Notch pathway.68 Loss of Shh or VEGF results in loss of arterial markers, whereas VEGF can rescue arterial differentiation in the absence of Shh signaling. The Notch pathway has also been reported to repress venous...
markers during the expression of artery-specific genes. Ephrins and their tyrosine kinase receptors (Ephs) are required for arterio-venous communications. Ephrin-B2, a membrane bound ligand with specificity toward the Eph-B4 receptor, marks arterial but not venous endothelial cells from the onset of vessel remodeling. However, Eph-B4 marks veins but not arteries. The null phenotype for each of these genes is similar, suggesting reciprocal coordination between these molecules in the formation of capillary beds. Finally, we have some knowledge of the molecular regulation controlling the formation of the lymphatic vasculature. The homeobox gene, Prox1, serves as a cue for the venous-derived lymphatic system, which is completely absent in mice null for this gene. A Prox1-positive subpopulation of cardinal vein endothelial cells are transformed into lymphatic vessels via a VEGF-C–dependent mechanism. Also, the developing lymphatic vasculature is disrupted by targeted inactivation of angiopoietin-2 in mice. Although several markers and stimuli of vessel morphogenesis are identified, much remains to be elucidated in the molecular regulation of these processes, particularly the responsible signaling pathways.

**Models of Vasculogenesis**

Although the vascular system remains a comparatively difficult "organ" to study developmentally because of its dispersed architecture and diverse cellular origins, model organisms, and culture systems characterized and developed mostly over the past 15 years have greatly accelerated our understanding of the molecular mechanisms ofvascular development. Vascular biologists now have many tools at their disposal—including, but not limited to, those described here.

**Whole Animal Models**

There is no question that whole organisms provide the most physiologically relevant systems in which to study vascular development. Vascular biologists have used a number of model organisms, each with distinct advantages, to discover the mechanisms of vasculogenesis. Avian embryos were used from the very beginning of vascular developmental biology and continue to be used today because of their experimental accessibility and amenability to elegant chimeric analyses, in combination with vascular and species-specific reagents such as the QH1 antibody. These experimental approaches have been further complemented by recent advances in viral cell labeling, which has greatly facilitated vascular cell lineage analysis, especially in the coronary vasculature.

**Embryonic Stem Cells**

Embryonic stem (ES) cells, especially those of murine origin, have revolutionized our ability to investigate the process of blood vessel development. They have been used to study not only the earliest stages of endothelial specification but also later morphogenetic processes in the primitive vasculature. In fact, vascular differentiation in the embryoid body (EB) system so closely recapitulates that of early in vivo vascular development that it has been suggested that phenotypes of genetic mutant animals can be predicted from the phenotypes of their cognate ES cells in culture. Furthermore, the accessibility of this system has allowed genome-wide gene-trap analyses to identify novel genes whose expression is confined to the developing vasculature.

In addition to the EB model of ES cell differentiation, ES cells cultured under tightly defined conditions to promote the growth of specific lineages have been used to support and study the role of lineage-specific precursors, especially hemangioblasts, during development. In fact, these same culture conditions have recently been used to determine that cells with potential to differentiate into both blood and endothelial cells exist in the posterior primitive streak of the early murine embryo. It is clear that the culture conditions (media, coated dishes, feeder cells, cystic embryoid body formation, etc) play important roles not only in the kinetics but also in the extent of endothelial differentiation in these systems. It is also important to note that these stem cell systems are devoid of blood flow and lack the spatial
organization of the developing embryo, perhaps limiting their usefulness as physiologically relevant models of later vascular biologic processes.

Conclusions

The experimental database that we use to understand endothelial differentiation and vascular development is exceptionally rich and draws on approaches that range from the imaginatively observational to the rigorously inductive. A number of key controversies in developmental biology have concerned the origin of endothelial cells and the means by which blood vessels are assembled. It has become obvious during the past decade that many of the principles of blood vessel development also apply to the assembly and disassembly of blood vessels in the adult, particularly in pathological circumstances. Activators and inhibitors of developmental pathways have been tested for their ability to modulate angiogenesis in early phase clinical trials, and in the case of anti-Flk1 antibodies clinical utility has been demonstrated for anti-tumor strategies.

Likewise, the explosion of interest in stem cell biology and the potential for regenerative medicine have caused many to reconsider the usefulness of understanding vascular developmental events with the notion that many of the pathways identified may be recapitulated in adult stem cells as they are coaxed toward the vascular lineage. Analyses of circulating endothelial progenitor cells, which have angiogenic potential, do indeed suggest that there are similarities in the biology of these cells compared with developmental endothelial precursors. Stem cell therapeutics therefore represents another potential arena for translation of insights from vascular development to clinical practice.

Even though our understanding of endothelial development is much richer than it was even a few years ago and despite the potential applications of this knowledge in clinical medicine, there are still a number of key issues on this topic that remain to be resolved. Precisely how early are endothelial precursors specified during development, and what is the nature of this progenitor cell pool? What are the relationships among signaling pathways that specify endothelial fates in a coordinated fashion? Is there a transcriptional hierarchy that regulates vascular development? The answers to these and other questions about endothelial development are likely to be forthcoming in the near future as experimental methods continue to evolve.

Acknowledgments

Work in the author’s laboratory is supported by National Institutes of Health (NIH) grants GM61728, HL65619, AG02482, and HL61656. C.P. is an Established Investigator of the American Heart Association and a Burroughs Wellcome Fund Clinical Scientist in Translational Research. J.F. is supported by an NIH-funded Integrative Vascular Biology training grant, T32-HL69768, and the UNC Medical Scientist Training Program.

References

6. Sabin FR. Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma, and red blood-cells as seen in the living chick. Anatomical Record. 1917;13:199–204.
Developmental Endothelial Differentiation

2253


Ferguson et al

Developmental Endothelial Differentiation

2253
Mechanisms of Endothelial Differentiation in Embryonic Vasculogenesis
J.E. Ferguson III, Rusty W. Kelley and Cam Patterson

Arterioscler Thromb Vasc Biol. 2005;25:2246-2254; originally published online August 25, 2005;
doi: 10.1161/01.ATV.0000183609.55154.44
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/11/2246

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/