Integrins in Vascular Development

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Abstract—In recent years, there has been a sustained interest in vascularization processes. Much, if not all, of the work has included the concept of new vessel morphogenesis. Surprisingly, most of the work has not addressed developmental mechanisms directly, but rather as an offshoot of a disease process, wound healing process, or from the perspective of inducing vessels in an ischemic site. One theme has dominated the various studies on capillary or endothelial tube morphogenesis—integrin-mediated cell behavior. Integrin biology impacts virtually every known step of nascent vessel formation. In this review article, we attempted to summarize key findings from the viewpoint of developmental biologists/morphologists. We also attempted to summarize and contrast data obtained using integrin gene ablation approaches in mice with other experimental systems. It is hoped this review will provide a distinct cell biological perspective to vascular scientists from the clinical, molecular, and tissue engineering communities. (Circ Res. 2001;89:566-572.)

Key Words: integrin ■ vascular morphogenesis ■ vasculogenesis ■ angiogenesis

The development of a vascular system is an early and critical event during vertebrate organogenesis. Vasculogenesis and angiogenesis are two mechanisms by which blood vessels develop. During vasculogenesis, a complex network of endothelial tubes in a precise spatial pattern is formed from mesoderm-derived cells before the initiation of blood flow. This process can be divided into several steps: (1) commitment of splanchnic mesoderm cells to an endothelial fate (angioblasts); (2) ventral repositioning of angioblasts into the splanchnopleural extracellular matrix (ECM); (3) formation of endothelial cordlike structures through cell extension/protrusive activity; (4) formation of vessel lumens; and (5) morphogenesis of large-caliber vessels through vascular fusion. Approximately 24 hours after circulation is initiated, vascular smooth muscle cells and pericytes are recruited to the endothelial tubes to form an increasingly mature vessel. For a more detailed overview of vasculogenesis, the reader is referred to several reviews.1-4 In contrast, angiogenesis is the formation of new vessels from preexisting vasculature by sprouting and intussusceptive microvascular growth.5-8

In both vasculogenesis and angiogenesis, vessel formation is a dynamic, complex biological process that depends on cellular interactions with regulatory factors and adhesive substrates. While the origins of vessel formation are different, virtually all molecules associated with one process are relevant to the other. These molecules include, but are not limited to, growth factors, growth factor receptors, ECM proteins, and cell-cell and cell-matrix receptors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiopoietins; VEGF receptors-1 and -2 (VEGFR1 and VEGFR2); fibronectin (FN); N-cadherin and VE-cadherin; α5β1, αvβ3, and αvβ5. Defects in a number of these molecules can result in embryonic death as demonstrated by gene ablation experiments9-15 or in adult patholog-
Integrins in General

Integrins are a family of noncovalently associated heterodimeric cell surface receptors composed of an α- and β-subunit that mediate cell-ECM and cell-cell adhesions. There are currently 18 α- and 8 β-subunits, not including splice variants, that combine to form more than 24 different integrins. Sixteen integrins are reportedly involved in vascular biology with 7 currently known to be expressed on endothelial cells (α(1), α(2), α(3), α(5), α(6), α(β), and α(β)) at varying times. Integrins are most widely known for their role as adhesion receptors for a variety of ECM proteins, such as FN, vitronectin, collagen, laminin, von Willebrand factor, fibrinogen, thrombospondin, and osteopontin. Most integrins recognize several ECM proteins and conversely, most matrix proteins bind more than one integrin. Integrins participate in many aspects of vasculogenesis, such as angioblast repositioning, extension/protrusive activity, lumen formation, and vascular fusion. Similarly, integrins are important for endothelial cell behavior during angiogenesis.

Regulation of Integrin Expression

The hypothesis that growth factor signaling regulates endothelial cell adhesive state is supported by a number of studies. Senger et al examining the response of human microvascular endothelial cells to VEGF stimulation, have shown an upregulation of integrins α(1), α(2), α(3), and α(5) in response to recombinant VEGF. Others have shown that VEGF enhances cell adhesion and migration mediated by α(5) and that other integrins involved in angiogenesis are activated, including α(1), α(6), α(7), and α(8). Addition growth factors, such as bFGF, are capable of regulating integrin expression on endothelial cells. Growth factors are not the only molecules to induce integrin expression and influence adhesion in vascular-related cells. Interactions between cells and their surrounding matrix environment can control expression of integrins at the mRNA and protein levels. Hypoxia induces differential expression of α(1) and α(5) in cultured human endothelial cells. Hypoxic conditions are known to stimulate production of VEGF, nitric oxide synthase, and nitric oxide in endothelial cells. As mentioned, VEGF has been implicated in regulating expression of α(1), α(5), α(6), and α(7) integrins. Recently it was shown that nitric oxide increased functional expression of α(1) on endothelial cells whereas an inhibitor of nitric oxide synthase significantly decreased expression.

Integrin Involvement in Matrix Assembly

In addition to their accepted role in cell attachment and migration through the ECM, integrins have been implicated in matrix assembly itself, as demonstrated most clearly with the αβ integrin and FN. Work by Fogerty et al suggests that the β1-subunit of the αβ integrin plays an important role in the binding and assembly of exogenous FN, more so than the α-subunit. This is supported by the fact that α-null embryos create a FN-rich matrix, indicating other integrins are involved or can compensate for the lack of αβ. This indeed has been confirmed with integrin αβ initiating FN matrix assembly in the absence of αβ, and FN matrix can be produced in the absence of both α- and β-subunits; however, it is markedly reduced. Cells lacking all β integrins are also capable of assembling FN matrices using the αβ integrin. Although it has been demonstrated that FN matrices can assemble in the absence of certain integrins, the data suggest matrix assembly is most efficient when cells are able to express their specific, entire repertoire of integrins.

Integrin Involvement in Vascular Lumen Formation

A number of in vitro models in collagen or fibrin matrices have demonstrated the importance of integrins in vessel lumen formation. In a three-dimensional collagen matrix, vacuole and lumen formation is completely dependent on integrin αβ interaction with collagen. On the other hand, integrin αβ, endothelial cell vacuole formation and coalescence into luminal structures are arginine-glycine-aspartic acid (RGD)-dependent and involve αβ and αβ integrins. Expression of antisense to the β1-integrin subunit or addition of anti–αβ and anti–αβ antibodies inhibited vacuole and lumen formation. How vessel lumens form in vivo is a complex puzzle that will be difficult to decipher, as vessels are forming in a milieu of matrix proteins whose composition differs both temporally and spatially.

Integrin Signaling in Vascular Cells

Integrin signaling is another crucial component in the development and remodeling of a vascular system. Through signaling, integrins can control gene expression, cell shape, proliferation, differentiation, and survival. These processes occur through outside-in signaling. Avidity modulation refers to agonist-mediated integrin clustering resulting in higher localized binding strength. What follows is a brief overview of integrin signaling as determined in vascular-related cells. For a more general and complete review of integrin signaling, see the reviews by Shattil and Ginsberg and Giancotti and Ruoslahti. Integrins are able to elicit signaling by associating with kinases through adapter proteins or as part of complexes with growth factor receptors. Receptor tyrosine kinases (RTKs)
can physically interact with integrins. Woodard et al.,40 using rat microvascular endothelial cells, studied the synergistic activity of platelet-derived growth factor (PDGF) receptor-β and integrin α5β1. PDGF-BB stimulated endothelial cell migration on vitronectin in an α5β1- and RGD-dependent manner with PDGF-Rβ and α5β1 coprecipitating. Similarly, phosphorylated VEGFR2 was found to coprecipitate with integrin α5β1.41 Addition of anti-αv and anti-β1 antibodies reduced phosphorylation of VEGFR2, phosphoinositide 3-OH kinase (PI3K) activity, cell migration, polarization, and proliferation.41 Both VEGFR2 and PDGF-Rβ associate with the extracellular domain of the integrin β3-subunit without ligand binding or phosphorylation of the RTKs.42

Integrin-mediated adhesion is capable of regulating gene transcription. Plating of endothelial cells on matrix proteins, β1- or α5-integrin antibodies induces transient phosphorylation in the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, with STAT5 mediating c-fos transcription.43 By an unknown signaling pathway, binding of capillary endothelial cells to varying concentrations of FN altered the mRNA expression and protein levels for the membrane-type 1 matrix metalloproteinase (MT1-MMP).44 MT1-MMP regulates the catalytic activation of MMP-2, which contributes to ECM degradation and, ultimately, cell shape, growth, and viability.44

Integrins are also implicated in cell survival. Endothelial cells bound to matrix proteins, β1-, or α5-integrin antibodies stimulated phosphorylation of the epidermal growth factor (EGF) receptor leading to the induction of the mitogen-activated protein kinase (MAPK) and cell survival.45 Specifically, the α5β1 integrin is implicated in endothelial cell survival in a number of studies. Ligation of α5β1 was found to be required for sustained MAPK/ERK activation by angiogenic growth factors.46 During angiogenesis, introduction of α5β1-integrin antagonists induced apoptosis in proliferating endothelial cells. Antagonists activated p53 and increased p21WAF1/CIP1 expression, a cell cycle inhibitor.47 The role of the α5β1 integrin in endothelial cell survival is supported by the work of Scatena et al.48 They demonstrated that endothelial cell survival on osteopontin and vitronectin required activation of nuclear factor (NF)-κB via the small GTP-binding protein Ras and tyrosine kinase Src. Inhibiting α5β1 ligation induced cell death by blocking NF-κB activity. Furthermore, fibronectin, laminin, and collagen type-I–induced cell survival did not require NF-κB, suggesting that cell survival can involve various integrins and signaling pathways.

Most cells express multiple integrins that are capable of simultaneously binding to many different ECM ligands. The process of coordinating the resulting integrin signals is called cross-talk. Integrins αvβ3 and αvβ6, expressed on human umbilical vein endothelial cells, were shown to undergo cross-talk in vitro. Ligation of integrin αvβ3 inhibited cell migration, mediated by αvβ6, toward FN.49 Conversely, evidence that αvβ3 can influence αvβ6 is provided by Varner and colleagues.50 Antagonists of αvβ3 suppressed αvβ3-mediated cell migration on vitronectin in vitro and angiogenesis in vivo by modulating protein kinase A activity.

**Gene Ablation Studies**

The importance of integrins to vascular development has been demonstrated by a number of in vivo studies, including both gene knockout and direct intervention. Several integrin subunits have been studied by gene knockout techniques with little evidence of abnormal vasculature, with the following four exceptions: (1) Mice deficient in α5 integrin have severe posterior trunk and extraembryonic mesodermal defects and die around embryonic day 10 to 11, possibly as a result of abnormal vessel formation and hemorrhaging.51 (2) The αv-knockout mouse has an interesting vascular phenotype. The majority of embryos die by midgestation; most likely as a result of placental defects. Those embryos that survive (~20%) die shortly after birth and exhibit extensive intracerebral and intestinal vascular abnormalities and hemorrhaging.52 Unexpectedly, all other vasculature appears normal. (3) Embryos lacking both αv- and α5-integrin genes produce an even more severe mesodermal defect than either one alone and die shortly after embryonic day 7.5.53 (4) Homozygous null embryos not expressing αv integrins are embryonic lethal as a result of two defects; the first is failure of fusion of the allantoids with the chorion and the second is a collection of defects in the epicardium and coronary vessels resulting in cardiac hemorrhaging.52

Similarly, the absence of specific integrin β-subunits may produce vascular defects. A viable and fertile mouse model having all of the characteristic features (prolonged bleeding times, cutaneous and gastrointestinal bleeding, and defects in platelet aggregation and clot retraction) of a human bleeding disorder, Glanzmann thrombasthenia, was generated by knockout of the β3-integrin gene.53 Loss of the β3-subunit disrupts the functions of the αmβ3 integrin on platelets and megakaryocytes and also αβ3 functions on a variety of cells. Reduced survival is observed as a result of defects in the maternal component of the β3-null placenta and through gastrointestinal hemorrhaging.53 In contrast to the intervention studies described in the next section, neovascularization of the mouse retina occurs in the absence of the β3 integrin.53 It has not been possible to discern the effects of the β3-subunit on vascular development by gene knockout, because lack of β3-integrin gene expression in mice has severe consequences: death occurs shortly after implantation.54,55

Alternatively, investigators have examined null embryos lacking integrin ligands. Those that follow have vascular defects. Fibronectin-null embryos are embryonic lethal (9th to 10th day of gestation) with defects in mesoderm, neural tube, and vascular development.13 Because a number of the integrin receptors (αvβ3, αvβ5, αvβ1, αvβ6, αvβ8, αvβ5, αvβ6, αvβ8, αmβ3) can use FN as a substrate, it is logical that absence of FN would have severe and wide-ranging abnormalities. Null embryos lacking one of the above receptors do not manifest as severe a phenotype as that of the FN knockout. Collagen I is important for the mechanical stability of developing vessels. Embryos lacking the ability to produce the α1(I) collagen chain, and thus type I collagen, die between embryonic days 12 through 14 because of a rupture of a major blood vessel.56 Similarly, mice lacking type III collagen have vascular defects. Although most type III collagen-deficient mice die shortly after birth, approximately 10% survive to
adults but have life spans that are one-fifth that of normal. Adult type III mutant mice die of blood vessel rupture, although the cause of neonatal death is unknown.

There are two obvious mechanisms that might complicate the interpretation of knockout data. One is the presence of compensatory mechanisms. This can be described as a process in which a different cell-ECM receptor substitutes for the missing integrin. The second is redundancy. This mechanism is commonly called on to explain why a mouse, missing a clearly important gene product, appears to be completely normal. Redundancy is normally envisioned as the availability of a backup system for a particularly important developmental mechanism. Thus, a different integrin receptor or some other extracellular matrix receptor is present on the cell surface (or immediately available) to substitute for the missing integrin-mediated adhesive activities. Arguably, the major difference between compensation and redundancy is that of the time scale. Presumably, compensatory mechanisms would entail a brief, finite period, when there was no specific integrin adhesive activity, followed shortly by the deployment of a functionally similar adhesive mechanism. Redundancy on the other hand, assumes that a backup system is immediately available.

Interventional Studies

An alternative method of investigating integrin function is to use inhibitory proteins, peptides, or explicitly designed small molecular perturbers in embryos and whole organisms. Agonists and antagonists can be introduced systemically via the circulation, whereas polymer-encapsulated or injected reagents can be introduced locally. The latter methods are well suited for examining locally affected areas where integrins have been perturbed, as well as examination of nearby regions where normal integrin-based cell behavior is available for comparison within the same embryo. In this regard, avian embryos are excellent subjects for examining vascularization de novo during early embryogenesis, as well as vessel formation in a physiologically mature circulatory system. Other experimental approaches include perturbation of vascularization in the chicken chorioallantoic membrane (CAM), grafting and chimeric models, rabbit corneal assays, surgical occlusion of vessels, introduction of an antibody, peptide, or small molecules into the circulation, whereas polymer-encapsulated or injected reagents can be introduced locally. The latter methods are well suited for examining locally affected areas where integrins have been perturbed, as well as examination of nearby regions where normal integrin-based cell behavior is available for comparison within the same embryo. In this regard, avian embryos are excellent subjects for examining vascularization de novo during early embryogenesis, as well as vessel formation in a physiologically mature circulatory system. Other experimental approaches include perturbation of vascularization in the chicken chorioallantoic membrane (CAM), grafting and chimeric models, rabbit corneal assays, surgically reduced circulation to a target region, and, of course, many different culture models of endothelial tube formation.

Cheresh and colleagues have conducted a large body of work demonstrating the involvement of α5 integrins in angiogenesis. These workers defined two distinct angiogenic pathways for integrins α5β2 and α5β1. Initial work found that expression of integrin α5β1 was increased at sites of active vessel formation. Addition of an inhibitory antibody to integrin α5β1 blocked angiogenesis induced by bFGF, tumor necrosis factor-α, and human melanoma fragments in CAM assays. Further work demonstrated that once angiogenesis was initiated by a tumor or cytokine, introduction of an α5β1 antagonist induced apoptosis of the proliferative vascular cells while leaving preexisting vessels unaffected. Disruption of integrin α5β1 ligand binding by antagonist increased p53 activity, increasing p21WAF1/CIP1 expression, therefore decreasing the bcl-2/bax ratio and promoting cell death.

In a SCID mouse/human chimeric model, an antagonist of α5β1 similarly inhibited progression of human breast cancer. Tumor growth and cell proliferation were decreased, as were the number of blood vessels. Furthermore, work by Brooks, Cheresh, and colleagues demonstrated an interaction between matrix metalloproteinase-2 (MMP-2) and integrin α5β1 localized to the surface of invasive cells. Disruption of the MMP-2/α5β1 interaction by PEX, a noncatalytic metalloproteinase fragment, or a small organic molecule (TSR1265) also inhibits angiogenesis and tumor growth in vivo.

In addition to roles in tumor angiogenesis, integrins α5β1 and/or α5β3 are implicated in other neovascular diseases, such as rheumatoid arthritis and degenerative or ischemic ocular diseases. Introduction of cyclic peptide antagonists of integrin α5β1 and α5β3 specifically inhibits new vessel formation in animal models of ocular neovascular diseases and arthritic disease but has no effect on established vessels.

Evidence that α5β3 is important in de novo blood vessel formation is provided by microinjection of antagonists into whole-mounted avian embryos. Injection of the anti-α5β3 monoclonal antibody LM609 at stages of active vasculogenesis disrupts the normal vascular pattern. Endothelial cells appear as clusters of round cells lacking normal cellular protrusions/extensions, leading to the failure of lumen formation. Although most work described thus far has dealt with α5 integrins, there is evidence for other integrin involvement. The role of β3 integrins has been similarly studied. Microinjection of CSAT, a β3-specific inhibitory antibody, arrests vasculogenesis in quail embryos at a stage when cordlike assemblies of endothelial cells rearrange to form tubes. Similarly, cyclic RGD peptides mimic the integrin antibody results; however, the effects are more pronounced and occur more rapidly.

Recently, a direct functional role for α5β3 integrin in neovascularization was demonstrated. Work in the laboratory of Varner and colleagues provides evidence that integrin α6β1 and fibronectin are coordinately upregulated in tumor vessels. Introduction of an antibody, peptide, or small molecule antagonist of integrin α5β3 blocks angiogenesis in chick and murine models. Furthermore, inhibition of tumor angiogenesis by the antagonist causes regression of human tumors in animal models.

Studies using ECM proteins and naturally occurring matrix fragments, which bind integrins, also implicate them as modulators of angiogenesis. For example, several domains of thrombospondin-1 contain peptide sequences capable of influencing angiogenesis. One such domain binds the α5β1 integrin and has been shown to modulate angiogenesis and endothelial cell behavior in vitro. Recently, noncollagenous (NC1) domains of specific type IV collagen chains (α2(IV), α3(IV), and α6(IV) NC1) have been shown to have potent antiangiogenic and tumor growth properties. Interestingly, integrin α5β1 binds to these α2(IV), α3(IV), and α6(IV) NC1 domains in an RGD-independent manner. The α2(IV) NC1 has also been shown to bind to integrin α5β3 with data suggesting a further interaction with α5β3. Endostatin, a cleavage fragment of collagen XVIII, is an endothelial-specific angiogenic inhibitor derived from tumors. Endostatin was found to interact with α5 and α6 integrins on endothelial cells.
In contrast to the antiangiogenic effectors above, developmental endothelial locus-1 (Del1) is a potent angiogenic ECM protein that promotes αβ3-dependent endothelial cell attachment and migration.78

Discrepancies Between Genetic and Nongenetic Studies

Despite considerable progress, marked discrepancies remain between reports of mouse mutants null for various integrins and the corresponding reports of studies in nongenetic experimental systems; however, some consistencies can be noted, as exemplified by work on β1 integrin loss of function experiments. As described above, mice null at the β1 locus die shortly after implantation54,55; similarly, introduction of β3-inhibitory antibodies causes widespread abnormalities in early avian embryos.59,79,80 With respect to the αβ3 integrin, however, it is difficult to reconcile the knockout mouse data with perturbational studies during avian vasculization. Why would inhibition of αβ3 integrin, by a single-dose injection of antagonist, cause a marked, reproducible alteration, whereas, in contrast, a mouse embryo null for the αv or β3-subunits exhibit normal vasculogenesis? Within an experimental embryo, what fundamental differences exist between a gene knockout approach and perturbation of a particular protein-protein interaction? Two differences immediately suggest themselves: one is the time at which “failure” or inhibition is first manifested; the second is the possibility that repair/recovery can take place in a perturbation experiment.

The case of a missing gene product can be likened to a process that does not begin at all; thus, the mechanism was incompetent from the beginning. In contrast, introduction of an inhibitor into an ongoing process can be likened to a breakdown of that process. Formation of endothelial tubes is ultimately a mechanical process: it is not difficult to imagine that if a mechanism is never employed (β3 knockout), the result is a different phenotype than if an ongoing β3-dependent process is disrupted.

With respect to the studies mentioned above, when an integrin gene product is missing there are commonly two outcomes observed: the first is that no meaningful perturbation is observed due, presumably, to rescue by a separate mechanism (eg, survival of β3 knockouts); the second outcome is overt failure of a developmental mechanism (eg, β3 knockouts). At the present state of technology, little of a dynamic nature can be learned from lethal vasculogenesis-stage mutations in mouse embryos (or, obviously, viable mutations). No data are available regarding the temporal progression of the lethal abnormality. In contrast, other embryonic systems are amenable to direct experimental observation. A fact that greatly facilitates understanding dynamic processes such as vasculogenesis (A. Czirok, P.A. Rupp, B.J. Rongish, and C.D. Little, unpublished data, 2001).

Early embryogenesis is characterized by wholesale change on a minute-by-minute, if not second-by-second, basis. In vertebrates, vasculogenesis occurs contemporaneously with somitogenesis, notochord extension, gastrulation, neurulation, and foregut formation; all-in-all a complex period. Vast alterations in cell position, shape, and proliferation typify each of these morphogenic processes, all of which require integrin-mediated cell adhesion. Any robust approach for understanding vasculogenesis will require a time-scale parameter. Tractable experimental systems such as zebra fish, frog, and avian embryos are amenable to time-lapse computational analysis within intact embryos. Such approaches allow systemic, global studies of the entire vasculogenic process.

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