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Minireviews

The Role of αv Integrins during Angiogenesis

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Mechanism of Angiogenesis

Angiogenesis or vascular remodeling depends on both growth factor stimulation and cell adhesion events. Angiogenesis and vascular remodeling not only contribute to development and wound repair but facilitate inflammatory disease, retinopathy, and cancer (1–3). For example, growth of solid tumors requires angiogenesis to supply oxygen, nutrients, and growth factors. The process of tumor-induced angiogenesis can be divided generally into three phases: initiation, proliferation/invasion, and maturation. First, angiogenic cytokines or growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or transforming growth factor α (TGF-α), are released from tumors and/or inflammatory cells (4). Second, these factors stimulate vascular cell proliferation and invasive behavior, promoting blood vessel growth and invasion of tumors. Growth factors and other angiogenic inducers bound to the extracellular matrix (ECM) can also be released upon matrix proteolysis, facilitating the vascular cell invasion phase. Finally, the invasive vascular sprouts deposit a basement membrane that facilitates differentiation and lumen formation (5–7). These basement membrane components maintain endothelial cells in a differentiated and quiescent state that is facilitated by cell–cell adhesive contacts (8). The newly formed vasculature not only provides nourishment for the tumor but also provides a conduit for metastatic cells to leave the primary site and metastasize to other locations (Fig. 1).

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Cell–Cell Interactions

Angiogenesis not only depends on growth factors but is also influenced by specific cell–cell and cell–ECM contacts. For example, antibodies to E-selectin, a transmembrane cell-adhesion glycoprotein, or sialyl-Lewis X or A, a cell-surface sialylated glycan, interfere with lumen formation in in vitro assays of blood vessel tube formation (8). Although E-selectin induces endothelial cell migration and angiogenesis in the rat cornea, E-selectin is neither a mitogen, nor does it influence endothelial cell mitogen production (9). In addition, a combined knockout of P- and E-selectin in mice does not result in blood vessel deformations (10).

Expression levels of immunoglobulin-like molecules, such as intercellular adhesion molecule 1 and vascular adhesion molecule 1 (VCAM-1), are induced in endothelial cells after stimulation with inflammatory cytokines [interferon γ (IFN-γ), interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), or lipopolysaccharides]. VCAM-1 induces angiogenesis in vivo and integrin α4β1-dependent migration of endothelial cells in vitro. VCAM-1 may act as an angiogenic stimulator through its interaction with α4β1, however, VCAM-1 does not affect endothelial cell proliferation. Another cell adhesion molecule, L1, an inducer of neurite outgrowth, has been shown to interact with the integrins αvβ3, αIIbβ3, αvβ1, or α5β1, present on platelets or endothelial cells via an RGD motif in the L1 molecule (11).

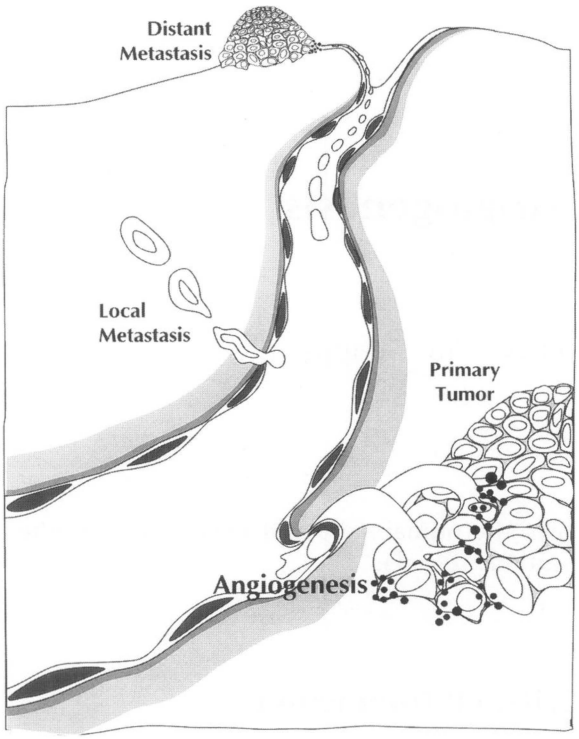

Fig. 1. Tumor-induced angiogenesis. Angiogenesis, the formation of new blood vessels from preexisting blood vessels during tumor-induced angiogenesis, can be divided into three phases. Angiogenic growth factors such as basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) are secreted from tumor cells. Tumor-secreted growth factors induce cell proliferation, cell invasion, expression of integrin $\alpha v\beta 3$ on vascular cells, and matrix degradation of surrounding extracellular matrix. The proteolytic degradation of the surrounding matrix microenvironment is a crucial step leading to new blood vessel growth and the maturation of the newly sprouted blood vessels.

Cell–Matrix Interactions

Cell adhesion to the extracellular matrix is mediated by integrins, which are heterodimeric transmembrane proteins that mediate cell–ECM interactions, and comprise a diverse family of over 15 α and 8 β subunits. Integrin subunits can heterodimerize in at least 20 different combinations. Different integrin combinations may recognize a single extracellular matrix (ECM) ligand, and other integrins may bind several different ECM proteins. Integrins are known to mediate not only the specificity of cell adhesion to a variety of matrix proteins but also the regulation of the cell cycle (12–14) and cell migration (15,16). Integrin ligation is known to induce a wide range of intracellular signalling events, including tyrosine phosphorylation of focal adhesion kinase and other focal contact-associated proteins (17), elevated intracellular pH and Ca^{2+} levels, inositol lipid synthesis, cyclin synthesis, and the expression of immediate early genes (12,13,18–22). Prevention of integrin interactions suppresses cellular growth or induces apoptotic cell death (2,23–25). Multiple integrins are expressed on endothelial cells, mediating adhesion to a wide variety of ECM proteins including fibronectin, vitronectin, laminin, collagen types I and IV, von Willebrand factor, fibrinogen, and denatured collagen (26).

Cell–Extracellular Matrix Interactions during Angiogenesis

Angiogenesis involves multiple interactions between the ECM and vascular cells. Dynamic remodeling of the extracellular matrix surrounding blood vessels facilitates several steps during angiogenesis, including matrix degradation and deposition of new ECM components. Laminin, a major component of basement membranes, is important during capillary tube formation (6,27). Various forms of collagen (types I, III, IV, and V) are also deposited during endothelial tube formation in vitro (28,29). The proteolysis of collagen can also influence endothelial tube formation since inhibition of matrix metalloproteinases (MMPs) block this process (30–32).

In vivo, ECM proteins are deposited during vasculogenesis and angiogenesis. For example, fibronectin is deposited followed by laminin during wound healing in the skin and in retinal and intraembryonal vasculogenesis (33–35). The role of collagens has been strongly implicated in angiogenesis through studies by Ingber and Folkman, who demonstrated that inhibition of collagen deposition and triple-helix formation, as well as inhibition of collagen cross-linking, prevented angiogenesis (36). Furthermore, function-blocking anti-$\alpha 1\beta 1$ and $\alpha 2\beta 1$ antibodies blocked VEGF-induced angiogenesis (37). De novo synthesis of collagen has also been shown to be important during angiogenesis (38,39). Genetic evidence for the role of collagen during angiogenesis is provided by a targeted gene knockout of collagen type I $\alpha 1$-chain, which resulted in the rupture of blood vessels, indicating defects in early blood vessel development (40).

In addition to collagen deposition, collagen degradation by MMPs has been implicated as an important step during the invasive stage of an-

giogenesis. For example, angiogenic inducers, such as bFGF, phorbal-12-myristate-13-acetate (PMA), and TNF-α, stimulate the expression of MMPs in endothelial cells (41,42). Tissue inhibitors of metalloproteinases (TIMPs), which regulate MMP activity on the cell surface, have been shown to inhibit angiogenesis in the chick embryo yolk-sac, chick chorioallantoic membrane (CAM), and rat cornea (42–44). The temporal and spatial coordination of collagen synthesis and collagen proteolysis in the ECM surrounding vascular cells is essential during angiogenesis. During the early phase of angiogenesis, local matrix degradation by MMPs may be required for invasion of pre-existing vascular cells through the basement membrane and surrounding stroma; this is followed by vascular cell migration and proliferation, which may depend on nascent expression and deposition of collagen. The proteolytic degradation of collagen may expose cryptic adhesive sites that are crucial for the invasive and, perhaps later, the migration phases of angiogenesis. For example, proteolysis of the intact collagen triple helix (types I and VI) can expose cryptic RGD-binding sites that are recognized by the integrin $\alpha v\beta 3$ (24,45,46). Integrin $\alpha v\beta 3$ is expressed preferentially on the surface of vascular cells undergoing angiogenesis in the chick CAM (25,47). Endothelial cells may use the cryptic RGD sites as a provisional matrix during the invasive and proliferative phases, providing a possible mechanism for coordinating adhesive and proteolytic activities during angiogenesis. Expression of collagen type IV and laminin during the deposition of new basement membrane may then facilitate differentiation and lumen formation during the later stages of angiogenesis (36).

αv Integrins and Angiogenesis

Of the wide spectrum of integrin subunit combinations that are expressed on the surface of cells, the $\alpha v\beta 3$ integrin has been identified as having an especially interesting expression pattern and distinct functional properties in vascular cells during angiogenesis. Integrin $\alpha v\beta 3$ is a receptor for a variety of ECM ligands with an exposed RGD moiety, including vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willebrand factor, osteopontin, and adenovirus penton base (16,48,49).

Integrin $\alpha v\beta 3$ has a very limited tissue distribution as it is not typically expressed on epithelial cells and only at low levels on intestinal, vascular, and uterine smooth-muscle cells (50). This receptor is also expressed on some activated leukocytes, macrophages, and osteoclasts, where it may function during bone resorption (51–53). Some invasive tumors such as metastatic melanoma (54) and late-stage glioblastoma (55) also express $\alpha v\beta 3$. Among the most prominent $\alpha v\beta 3$ expression levels have been identified in cytokine-activated endothelial or smooth muscle cells, especially on blood vessels in granulation tissue and tumors (25,47,56,57). The expression of $\alpha v\beta 3$ on blood vessels suggests that it may be important for vascular cell proliferation and for migration events associated with restenosis and angioplasty. The vascular smooth-muscle cell migration that occurs during restenosis is dependent on $\alpha v\beta 3$ ligation and is accompanied by increased expression levels of osteopontin, an $\alpha v\beta 3$ ligand (58). A function-blocking anti-$\beta 3$-antibody has been shown to be beneficial in high-risk angioplasty patients (59). In addition to the possible role of $\alpha v\beta 3$ during restenosis, there has been a significant advance in the understanding of the possible role of integrin $\alpha v\beta 3$ during angiogenesis and vascular remodeling in recent years.

Integrin $\alpha v\beta 3$ is expressed at low level in quiescent or normal blood vessels, but it is induced following exposure to cytokines, growth factors, or tumors (47). bFGF induces $\beta 3$ mRNA and surface expression on cultured human dermal microvascular endothelial cells (57,60). The bFGF-induced increase in $\alpha v\beta 3$ mRNA has been shown to be dependent on the nascent expression of the *Hox* D3 homeobox gene followed by $\alpha v\beta 3$ expression (61). Furthermore, $\alpha v\beta 3$ protein expression is induced by bFGF on blood vessels in the chick CAM (47) and on the rabbit cornea (62). $\alpha v\beta 3$ expression in vascular cells is also induced by human tumor cells cultured on the CAM (25,47) and during wound healing (47), macular degeneration, diabetic retinopathy, and other neovascular diseases of the eye (63).

The up-regulation of $\alpha v\beta 3$ during angiogenesis suggests that integrins may have an important function during angiogenesis. In fact, disruption of integrin $\alpha v\beta 3$ ligation with antibody (LM609) or cyclic peptide antagonists of $\alpha v\beta 3$ prevent blood vessel formation in the chick CAM, quail embryo, rabbit cornea, mouse retina, and in human skin transplanted onto severe combined immunodeficiency disease (SCID) mice (47,62,64–66). Angiogenesis induced by

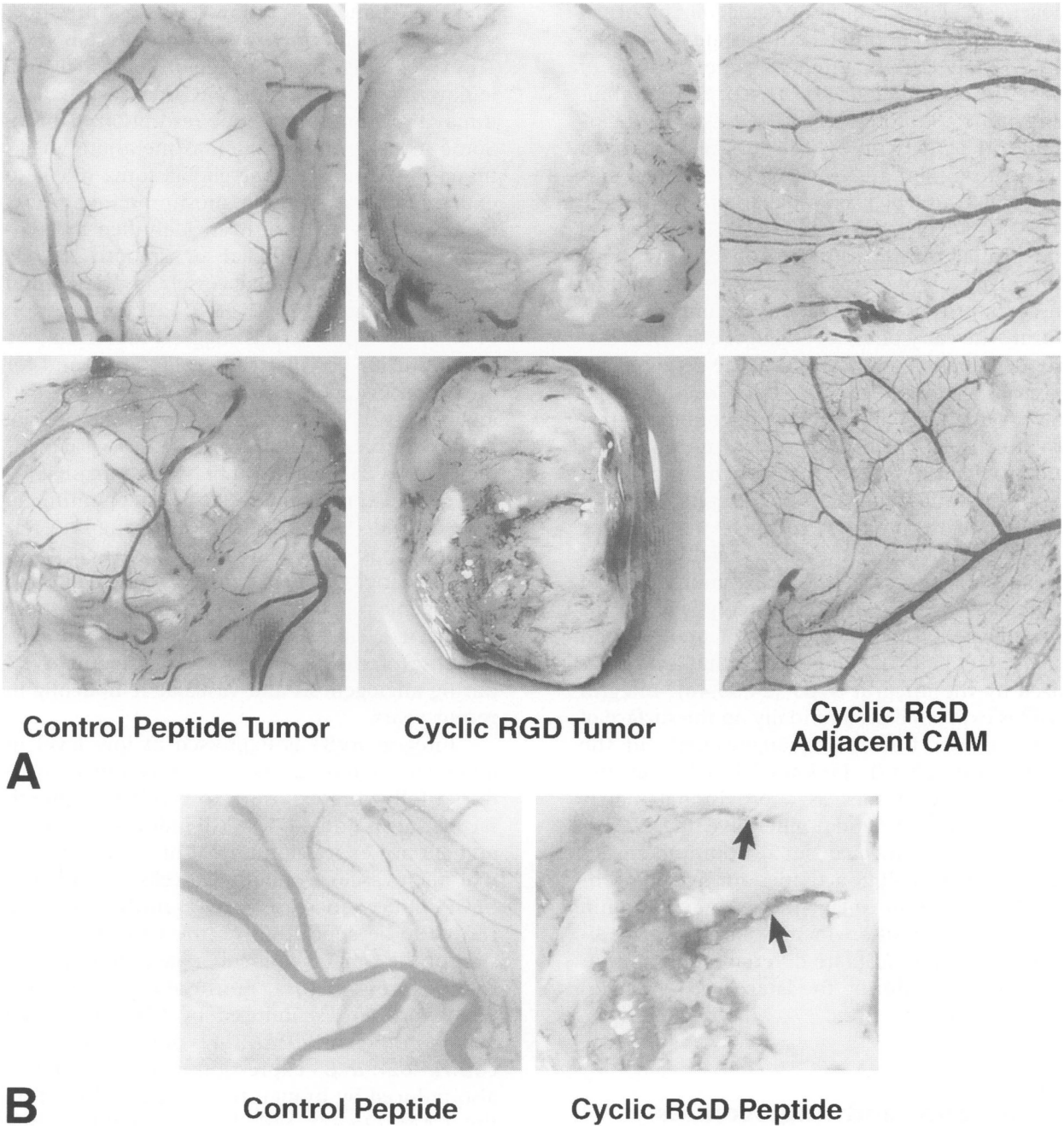

Fig. 2. Tumor-induced angiogenesis blocked by integrin αv antagonists in the chick chorioallantoic membrane (CAM) angiogenesis model (A) M21L human tumor cell fragments lacking $\alpha v\beta 3$ or $\alpha v\beta 5$ were inoculated on the surface of the CAM of a 10-day-old chick embryos. After 24 hours, embryos received intravenous injections of either control RADfV cyclic peptide (left) or RGDfV cyclic peptide (middle). Adjacent CAM tissue from cyclic peptide is treated CAM is shown on the right. After three days CAMs were excised and analyzed for vascularization. (B) High magnification of the peptide-treated tumors. The left panel is a control peptide-treated CAM and on the right is a cyclic RGD peptide-treated CAM. Note that the αv antagonists acted on the CAM vessels decreasing vascularization of the tumor, since the tumor cells do not express $\alpha v\beta 3$ or $\alpha v\beta 5$ (67).

$\alpha v\beta 3$-negative human tumor cells was also blocked with $\alpha v\beta 3$ antagonists (Fig. 2). These antagonists prevented the growth of new blood vessels without detectably influencing the preexisting blood vessels. Furthermore, the inhibition of blood vessels supporting tumors not only

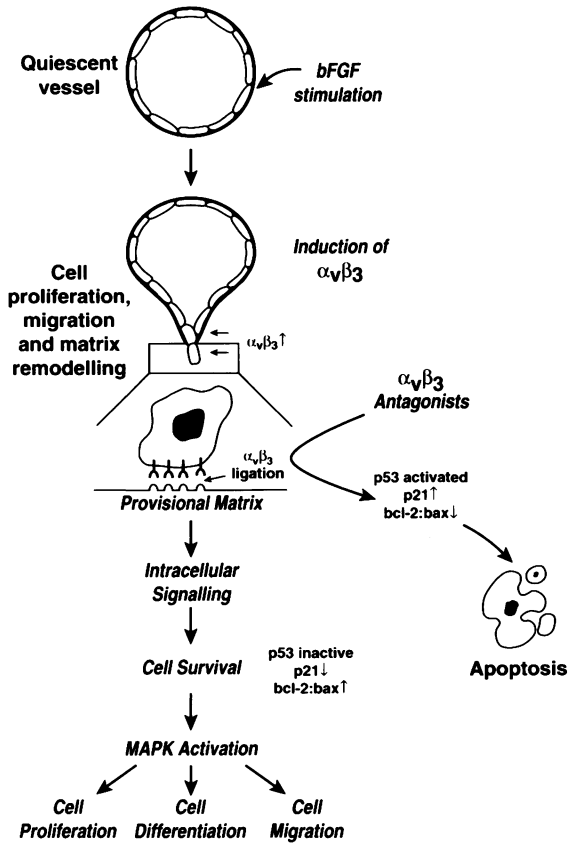

Fig. 3. Hypothetical model for the role of integrin $\alpha v\beta 3$ in angiogenesis. Angiogenic stimulus by basic fibroblast growth factor (bFGF) induces expression of $\alpha v\beta 3$ and causes cells to invade the surrounding extracellular matrix (ECM) and to enter the cell cycle. When ligation of integrin $\alpha v\beta 3$ is blocked, proliferating vascular cells undergo apoptosis, accompanied by an increase in p53 activity, $p21^{WAF1/CIP1}$ expression, and a decrease in the Bcl2:Bax ratio. bFGF induces the activation of mitogen-activated protein kinase (MAPK) in vascular cells and leads to cell proliferation, differentiation, and migration.

blocked tumor growth but induced tumor regression (25). Histological examination of tumors treated with the $\alpha v\beta 3$ antagonists revealed few if any viable tumor cells or detectable blood vessels (25). Cytokine or tumor cell–stimulated blood vessels treated with the $\alpha v\beta 3$ antagonists have been shown to undergo programmed cell death (apoptosis) in response to administration of the antagonists (47). These findings suggest that integrin $\alpha v\beta 3$ can provide specific cell survival signals that facilitate vascular cell proliferation during angiogenesis (Fig. 3).

A genetic approach to examining the role of the αv and the $\beta 3$ integrin subunits has provided some insight into the role of these molecules during vasculogenesis and angiogenesis. In Glanzmann thrombobastemia patients, who lack functional $\beta 3$ integrin protein (68,69), there appears to be normal development of blood vessels, although the wound-healing response is associated with extensive bleeding. This suggests that although functional $\beta 3$ levels are diminished, perhaps the expression of integrin $\alpha v\beta 5$, another RGD-specific integrin, may compensate for the absence of functional $\alpha v\beta 3$. In fact, integrin $\alpha v\beta 5$ can potentiate a distinct pathway of VEGF-induced angiogenesis (62).

Genetic knockout experiments of the αv gene in mice have shown that some mice survive to term, but die shortly after birth (70). These αv-null mice have extensive brain and intestinal hemorrhaging, which indicates a defect in blood vessel integrity demonstrating the requirement for the expression of αv integrin in blood vessel formation and/or maturation. Other organs have apparently normal vascularization (70), suggesting that animals lacking αv integrins can compensate to some degree by perhaps utilizing other integrin-dependent adhesion pathways. Interestingly, mice lacking the $\beta 3$ subunit while having a bleeding disorder contain normal brain and intestinal blood vessels (71). Therefore, if one considers that brain and intestinal blood vessel development is disrupted by an αv knockout, an integrin other than $\alpha v\beta 3$ must be required for blood vessels in these organs (Table 1).

Integrin $\alpha v\beta 3$ and Vascular Cell Survival

Systemic administration of $\alpha v\beta 3$ antagonists has been shown to induce apoptosis in cytokine or tumor cell–activated blood vessels (25). In fact, primary endothelial cells are known to be anchorage-dependent for growth and can undergo apoptosis when treated with anti-integrin function-blocking antibodies (23,75). These results suggest that ligation of $\alpha v\beta 3$ on vascular cells may mediate a signaling event that is essential for the survival and differentiation of vascular cells undergoing angiogenesis in vivo. Using DNA laddering and measurements of free 3'OH groups from fragmented DNA as markers of apoptosis in endothelial cells, $\alpha v\beta 3$ antagonists induced apoptosis after 48 hr of treatment (25). The $\alpha v\beta 3$ antagonists

Table 1. Summary of αv integrin requirements during angiogenesis and in knockout mice during development

Model	Evidence for a role of αv integrins in angiogenesis (reference)
Inhibitor Studies of αv Integrin Antagonists (antibodies, peptides, organics)	
Chick	Block growth factor-induced angiogenesis on the CAM (47)
	Causes regression of human tumors on the CAM (25)
Mouse	Inhibit
	Retinal vessel outgrowth (63)
	Human tumor xenographs (72)
	Human/SCID chimera tumors (64)
Rabbit	Inhibit
	Corneal micropocket angiogenesis (62)
	Arthritic knee angiogenesis, thereby reducing disease (73)
	Image tumors with antibody-coated liposomes as detected by MRI (74)
Knockout Studies	
αv knockout	Lethal because of defective brain and intestinal vessels (70)
β3 knockout	No vascular defect suggests an αv integrin other than αvβ3 is required for brain and intestinal vessels (71)

administered during angiogenesis in the chick CAM also induced endothelial cell, p53-dependent DNA-binding activity, which regulates the cell-cycle inhibitor $p21^{WAF1/CIP1}$ (76). In cultured endothelial cells, ligation of αvβ3 with immobilized antibody reduced the p53 activity and subsequent $p21^{WAF1/CIP1}$ protein levels. Ligation of αvβ3 was also shown to increase the expression of Bcl-2 and decrease Bax levels in primary endothelial cells, resulting in an increase in the Bcl-2:Bax ratio (75), a signal known to promote cell survival (77). Therefore, ligand binding by endothelial cell αvβ3 may suppress apoptosis and conflicting growth-arrest signals, facilitating the proliferation and differentiation of new blood vessels (Fig. 3). It is important to point out that mice clearly survive without p53, indicating that alternative or compensatory apoptotic mechanisms can allow normal development. The fact that αv integrin antagonists activate a p53-dependent cell death pathway suggests that mice lacking p53 may also not depend on αv integrins for vascular development. This in turn may account for the presence of blood vessels in mice lacking αv integrins.

Regulation of MAP Kinase Activity in Angiogenesis

Angiogenesis is initiated by growth factors and depends on cell adhesion events to mediate the signaling events that lead to vascular cell proliferation, migration, and differentiation. Integrins and growth factor receptors have been co-localized on the cell surface and appear to cooperate in their capacity to activate the ras/mitogen-activated protein (MAP) kinase pathway (78,79). Although growth factors can initiate MAP kinase activity and vascular cell proliferation, it is not clear how these signals ultimately lead to vascular remodeling events, which involve endothelial cell invasion and lumen formation of angiogenic sprouts in vivo. We have recently identified two bFGF-induced ERK activation signals required for angiogenesis in vascular cells (67). One is immediate and not influenced by integrin antagonists, and a second sustained signal is αvβ3-dependent. Both the immediate and the sustained activated ERK was localized to vascular cells in the chick CAM model of angiogenesis. Using a synthetic inhibitor of the MAP kinase pathway, we demonstrated that active MAP kinase is re-

quired during angiogenesis in vivo. Previous studies have suggested that the duration of intracellular signaling events profoundly influences whether a cell undergoes proliferation and/or differentiation (reviewed in ref. 80). For example, exposure of PC12 cells to epidermal growth factor induces a rapid and transient MAP kinase activity leading to cell proliferation, while nerve growth factor induces cell differentiation due to a sustained MAP kinase activation (81).

It is possible that during angiogenesis, ligation of $\alpha v\beta 3$ through appropriate matrix contacts provides the positional or molecular cues necessary for sustained ERK activity. This may allow prolonged cell survival and differentiation of only those cells that are in the appropriate matrix microenvironment. In fact, antagonists of $\alpha v\beta 3$ caused apoptosis of proliferating vascular cells undergoing angiogenesis in the chick CAM, and this was associated with increased endothelial cell p53 activity. Interestingly, MAP kinase activity can regulate both p53 activity (82) and cell survival (76) in vitro. These observations indicate that sustained activation of ERK by $\alpha v\beta 3$ ligation during angiogenesis may suppress p53 activity, thereby promoting vascular cell survival and the maturation of newly sprouting blood vessels (Fig. 3).

Two pathways of angiogenesis have recently been identified, based on their dependence on the related but distinct integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (62). In studies in both the rabbit corneal eye pocket and CAM angiogenesis assays, anti-$\alpha v\beta 3$ monoclonal antibody antagonists blocked bFGF-induced angiogenesis, whereas anti-$\alpha v\beta 5$ antagonists blocked VEGF-induced angiogenesis. Furthermore, inhibition of the PKC pathway blocked the VEGF-induced angiogenesis specifically but did not affect bFGF-induced angiogenesis. These distinctions between these individual pathways of angiogenesis suggest that further examination of the specific signaling requirements of specific growth factors during angiogenesis may lead to a better understanding of the growth factor requirements during tumor-induced angiogenesis.

Future Perspectives

The elucidation of the molecular basis of angiogenesis remains a challenge because of the complex ECM–vascular cell interactions that must be temporally and spatially coordinated. Further examination of the signaling events transduced by cell adhesion molecules to the smooth muscle and endothelial cells will probably reveal mechanisms by which cells process cytokine or growth factor stimuli to effect changes in intracellular phosphorylation cascades, gene expression levels, and ECM-associated enzymatic activities. The coordinated response to these inputs may direct the processes of vascular cell invasion, migration, proliferation, and differentiation during angiogenesis.

Clinical trials are now ongoing to examine the effects of a humanized form of the anti-$\alpha v\beta 3$ antibody, LM609 (Vitaxin) (Table 1). This antibody is being administered to late-stage cancer patients in a dose-escalation study. A second trial is now underway using a small cyclic peptide antagonist of both $\alpha v\beta 3$ and $\alpha v\beta 5$. Initial clinical results show these antagonists are not only safe but may be providing clinical benefit in a number of patients.

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