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Role of Integrins in Angiogenesis

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INTRODUCTION

ANGIOGENESIS, OR the development of new blood vessels from pre-existing vessels, is a tightly regulated process that plays a critical role in a variety of normal physiological events, including trophoblast implantation, wound healing and embryonic development [1–4]. However, uncontrolled neovascularisation can contribute to a number of pathological processes such as rheumatoid arthritis, diabetic retinopathy and tumour growth and metastasis [1–4]. Therefore, the identification of molecules that regulate angiogenesis, and in turn, understanding how these molecules function during the angiogenic cascade, are major concerns facing researchers in the field of modern vascular biology.

THE ANGIOGENIC CASCADE

Angiogenesis requires the co-operation of a variety of molecules that regulate cellular processes such as extracellular matrix (ECM) remodelling, invasion, migration and proliferation. For simplicity, angiogenesis can be organised into three generalised stages including an initiation phase, a proliferative/invasive phase and a differentiation/maturation phase (Figure 1). However, it is important to point out that while these are distinct cellular processes, they do not occur in isolation, but rather are interconnected in a continuum of biological events. The initiation phase of angiogenesis can be accomplished by the activation of vascular cells by a variety of angiogenic cytokines and other physiological mediators. A partial list of the more well-characterised growth factors known to promote angiogenesis include basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived endothelial-cell growth factor (PDECGF) and tumour necrosis factor alpha (TNF-α) [5, 6]. These cytokines and other angiogenic molecules can be released from a number of sources including inflammatory cells, mast cells, macrophages as well as a variety of tumour cells.

These cytokines activate the normally quiescent vascular endothelium by binding to their respective cell surface tyrosine kinase receptors. Some of these receptors are expressed predominantly on endothelial cells such as the two recently described VEGF receptors termed Flt-1 (fms-like tyrosine kinase-1) and Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1) [7, 8]. A third tyrosine kinase receptor called Flt4 is structurally related to these receptors, but it appears to be associated primarily with lymphatic endothelium [9]. In addition to the VEGF receptors, two other endothelial specific tyrosine kinase receptors have been identified and termed Tie-1 and Tie-2 (TEK), but their physiological ligand remains unclear [10]. Recently, studies involving gene knockout mice have implicated these VEGF receptors and Tie-1 and Tie-2 in biochemical signalling events necessary for angiogenesis and vascular development [7, 8, 10].

As mentioned previously, binding of endothelial specific tyrosine kinase receptors can lead to the activation of vascular endothelial cells. In turn, these activated endothelial cells have a characteristic set of traits which include increased cellular proliferation, elevated expression of cell adhesion molecules, increased secretion of proteolytic enzymes, and increased cellular migration and invasion. These complex cellular processes help promote the growth and invasive stages of the angiogenic cascade (Figure 1). In this regard, a number of distinct molecules have been suggested to promote cellular proliferation and invasion, including members of the integrin, selectin and immunoglobulin supergene families [11–13]. Moreover, recent studies also suggest that invasive cell behaviour depends not only on cell adhesive mechanisms, but requires functional co-operation between adhesive molecules and proteolytic enzymes [14, 15]. In this regard, a variety of matrix degrading proteases have been suggested to contribute to the invasive behaviour of newly sprouting blood vessels, such as members of the matrix metalloproteinase and serine proteinase families [16, 17]. Eventually, the activated endothelial cells invade and migrate through the proteolytically altered micro-environment and begin to align themselves into vascular cords. Finally, a complex series of biochemical signals, presumably derived from cell surface receptors interacting with ECM components and/or other soluble factors, result in lumen formation and differentiation into mature blood vessels (Figure 1).

EXTRACELLULAR MATRIX AND ANGIOGENESIS

It is well known that the ECM can play an important role in regulating cellular behaviour. In fact, it has been suggested that changes in ECM composition due to either altered secretion of ECM components and/or extensive proteolytic remodelling can modulate cell–ECM interactions [18–20]. In this regard, vascular cells must have the capacity to sense and in turn respond to the continuum of changes that occur in the composition and structure of the vascular ECM during angiogenesis. One group of molecules well sui-

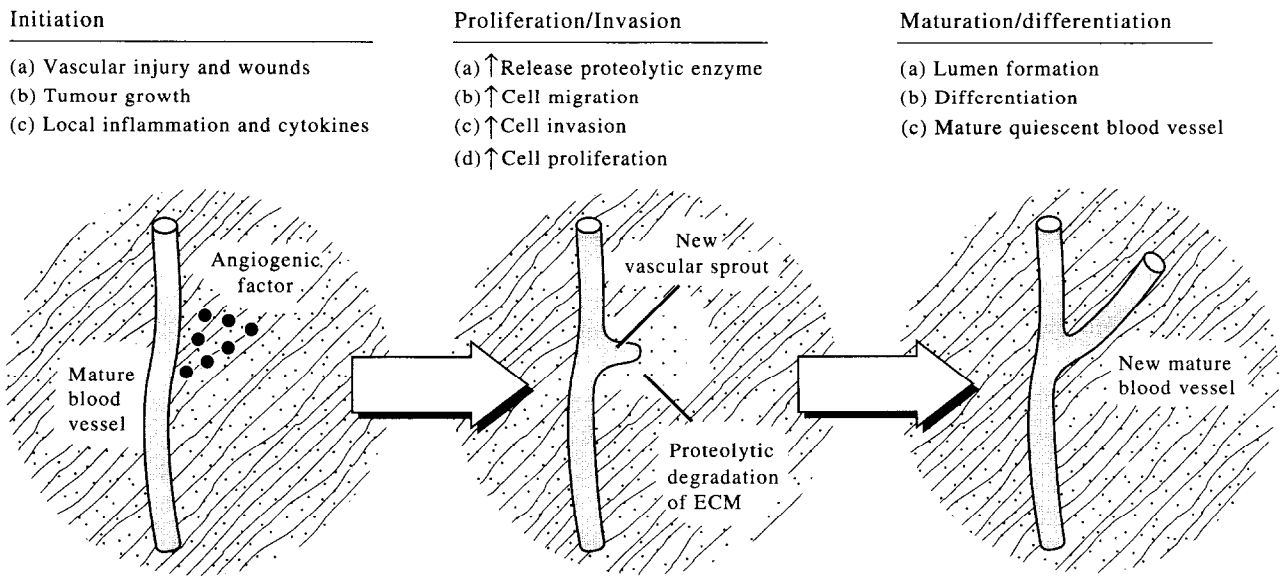

Figure 1. The angiogenic cascade. Angiogenesis can be described as a continuum of three phases including an initiation phase, a proliferation/invasive phase and a maturation/differentiation phase. Angiogenesis can be initiated by a number of mechanisms including vascular injury, tumour growth and activation by a variety of cytokines. Proliferative/invasive phase: this stage is characterised by increased release of proteolytic enzymes, differential regulation of cell adhesion molecules, and increased migration, invasion and proliferation. Maturation/differentiation phase: cell–cell and cell–ECM interactions may lead to a variety of biochemical signals resulting in differentiation into mature blood vessels.

ted for such a task is the integrin class of cell adhesion receptors. Numerous reports have demonstrated the ability of integrins to play a critical role in mediating cell–ECM interactions [12, 21]. In recent years, a growing body of evidence has suggested a critical role for integrin receptors in the regulation of angiogenesis and vascular development. Therefore, in this report, we will focus primarily on the roles of integrins in this complex biological process.

THE INTEGRIN FAMILY OF CELL ADHESION MOLECULES

Integrins are a family of multifunctional cell adhesion molecules composed of non-covalently associated α and β chains. To date, at least 15 α and 8 β integrin subunits have been identified [12, 21]. These integrin subunits can combine to give a wide variety of heterodimers with distinct cellular and adhesive specificities. The unique combinations of α and β chains dictates, in part, their ligand specificities. These transmembrane ECM receptors can bind to a number of ECM components including fibronectin, laminin, collagen, vitronectin, fibrinogen and thrombospondin. Importantly, many of these ECM molecules help compose the complex architecture of the surrounding vascular matrix and subendothelial basement membrane [22, 23]. It is important to point out that while these ECM molecules are known components of the vascular microenvironment, their expression is temporally and spatially regulated during the angiogenic cascade [24–27]. Thus, the levels of expression, distribution and integrity of a particular matrix protein at any one time during the angiogenic cascade may change, leading to significant variations in the ECM protein composition. In fact, it has been suggested that the provisional matrix associated with blood vessels during the early invasive phase of angiogenesis includes increased levels of interstitial type-I collagen and fibronectin [22–27]. In contrast, the latter differentiation and maturation phases of angiogenesis are characterised by increased secretion and deposition of basement membrane collagen type IV and laminin [22–27]. Thus, the possibility exists that cell type specific recognition of individual matrix components by integrin receptors may regulate distinct cellular events during angiogenesis.

Integrins, in general, have been shown to regulate a variety of cellular responses such as adhesion, migration, invasion, proliferation and recently, cell survival and apoptosis [28–30]. Thus, these receptors are not simply glue that binds cells to the ECM, but rather multifunctional molecules capable of transmitting biochemical signals from the ECM to the cells interior. In fact, it has been shown that the cytoplasmic tails of integrin subunits can interact with specific components of the cytoskeleton such as α-actinin, talin and vinculin [31, 32]. These distinct protein–protein interactions may potentiate a number of transmembrane signalling events. In this regard, recent reports have indicated that integrin binding can lead to fluxes in intracellular calcium, changes in intracellular pH, protein phosphorylation and regulation of gene expression [33, 34].

CELLULAR DISTRIBUTION OF INTEGRINS

The integrin family of receptors are expressed on a wide range of cells. While specific members, such as the β2 integrins, have a somewhat restricted distribution to leucocytes and some cells of haemopoietic origin, other members, including β_1, β_3, β_4 and β_5, are more widely distributed [12, 28]. Numerous studies have demonstrated expression of the β_1 integrin subfamily members on endothelial cells including $\alpha_1\beta_1$, $\alpha_2\beta_1$ $\alpha_3\beta_1$, $\alpha_4\beta_1$ $\alpha_5\beta_1$ and $\alpha_6\beta_1$ [21, 35]. In addition, members of the β_3, β_4 and β_5 subfamilies have also been shown to be expressed on endothelial cells. However, while many integrins are consititively expressed, others have been shown to be differentially regulated. In fact, studies by Enenstein and coworkers have shown that the angiogenic cytokines bFGF and transforming growth factor-β (TGF-β)

can differentially modulate the expression of specific integrins in cultured microvascular endothelial cells *in vitro* [36]. For example, bFGF treatment causes the increased expression of α_2, α_5, β_1 and β_3 integrins, whereas TGF-β stimulation increases the synthesis of α_2, α_5 and β_1 but not β_3 [36]. Moreover, Sepp and colleagues and Swerlick and associates also showed increased β_3 expression upon bFGF stimulation of cultured endothelial cells [37, 38]. When similar studies were performed *in vivo,* Enenstein and associates reported that α_2 and α_v integrins were highly expressed on the tips of sprouting angiogenic blood vessels [39]. Recently, we have shown that integrin $\alpha_v\beta_3$ is only minimally expressed on quiescent blood vessels, but is significantly upregulated during angiogenesis *in vivo* [40]. In fact, a significant increase in $\alpha_v\beta_3$ expression was observed on both human and chick blood vessels after stimulation with recombinant cytokines or fragments of solid human tumours [40, 41]. Finally, Clark and associates confirmed these findings in a model of porcine wound healing and showed that $\alpha_v\beta_3$ was highly but transiently expressed on invasive capillary sprouts during granulation tissue formation [42]. However, the simple surface expression of a given integrin does not necessarily prove that integrins plays a functional role in angiogenesis. Therefore, numerous investigators have begun studying the functional roles of the integrin receptor in blood vessel development in more detail.

β_1 INTEGRINS AND ANGIOGENESIS

Currently, there are no *in vitro* models of angiogenesis that completely mimic the complex cellular and biochemical processes that occur during angiogenesis *in vivo.* However, *in vitro* endothelial tube forming assays have been developed which may approximate certain cellular events during angiogenesis. In this regard, Bauer and coworkers utilised an *in vitro* cord forming assay to study the role of integrins in this process [43]. Monoclonal antibodies (MAb) directed to either α_6 or β_1 integrin subunits blocked endothelial cord formation *in vitro,* whereas an antibody to α_5 integrin had little if any effect [43]. These findings suggest a role for β_1 integrins and in particular, $\alpha_6\beta_1$ integrin, in cellular events involved in new blood vessel development.

In similar studies, Davis and colleagues used polyclonal antibodies directed to the fibronectin receptor $\alpha_5\beta_1$ and MAbs to the β_1 subunit to inhibit endothelial cord formation *in vitro* [44]. These findings were consistent with reports by Bauer and coworkers, again suggesting a role for β_1 integrins in angiogenesis. However, one must use caution in extrapolating results from *in vitro* tube forming assays, since these models may not completely mimic the complex cellular processes that occur *in vivo.* In fact, recent studies by Yang and associates showed that α_5 deficient mice had numerous vascular defects, suggesting an important role for α_5 integrin in vascular development [45].

Further evidence supporting a role for β_1 integrins in angiogenesis has been provided by the studies of Grant and colleagues. Antibodies directed to the β_1 laminin receptor and synthetic peptides derived from distinct regions within laminin disrupted endothelial cell differentiation and capillary-like tube formation *in vitro* [46]. Interestingly, recent findings by Schnaper and colleagues have identified specific regions of laminin that may potentiate distinct endothelial cellular behaviour [47]. For example, while the RGD sequence within laminin appears to regulate predominantly cell adhesion, the YIGSR and SIKVAV sequences regulate cellular morphology, differentiation and cell migration, respectively [47].

In addition to laminin, another ECM molecule that is abundantly expressed in the vascular micro-environment is collagen. Davis and colleagues have used three-dimensional collagen matrices to study the role of the collagen receptor α2β1 in capillary lumen formation [48]. These findings suggest that capillary lumen formation requires the formation of endothelial cell vacuoles [48]. In particular, MAbs directed to either α_2 or β_1 integrin subunits block vacuole and lumen formation *in vitro,* while antibodies directed to other integrins such as α_3, α_5 or α_6 have no effect [48]. Interestingly, while α_5 and α_6 have been clearly implicated in vascular development, they are apparently not involved in the specific events necessary for lumen formation *in vitro.* Alternatively, α_5 and α_6 integrins may regulate interaction with fibronectin or laminin that may be necessary for cellular migration and invasion within the vascular micro-environment. These studies demonstrate the importance of understanding the specific cellular and biochemical processes that are being regulated by distinct vascular integrins at specific points during the angiogenic cascade.

Finally, in studies by Drake and colleagues, antagonists of β1 integrins were micro-injected into quail embryos resulting in significant disruption of vascular development and lumen formation [49]. While these studies focused primarily on the distinct process of vasculogenesis rather than angiogenesis, it does provide critical evidence for a role of β_1 integrins in vascular development *in vivo.*

POSSIBLE ROLES FOR β_1 INTEGRINS IN ANGIOGENESIS

It appears from the studies discussed above that several β_1 integrins may play an active role in angiogenesis. However, which specific cellular and biochemical processes are regulated by these integrins during angiogenesis is not clear. Therefore, it is of interest to speculate on the possible mechanisms by which these β_1 integrin, in co-operation with specific ECM components, might contribute to the angiogenic cascade. In this regard, it is known that β_1 integrins can potentiate endothelial cell interactions with a variety of ECM components including fibronectin, laminin and collagen. Therefore, it is possible that integrin binding of ECM proteins, such as collagen, may regulate blood vessels morphogenesis and structural integrity. This contention is supported by the fact that collagen is a major component of the vascular ECM and has been suggested to play an important role in the structural integrity of blood vessels [22, 25, 50]. In fact, mice defective in collagen type I expression are characterised by numerous vascular defects [51]. In addition, studies by Ingber and colleagues and Haralabopoulos and associates demonstrate that antagonists of collagen synthesis and deposition can significantly inhibit angiogenesis [52, 53]. Thus, it is possible that $\alpha_2\beta_1$, a known collagen receptor, may regulate cell adhesion, migration, and perhaps lumen formation, all events thought to be necessary for angiogenesis. Moreover, β_1 integrins are also known to mediate cellular interaction with basement

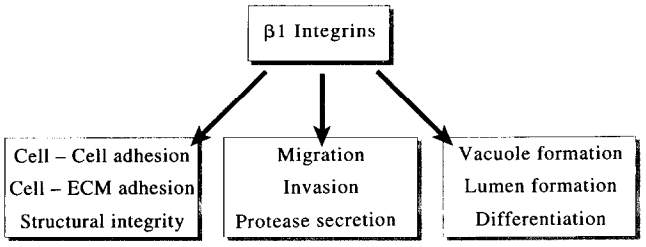

Figure 2. Potential roles for β_1 integrins during angiogenesis. A number of distinct β_1 integrins have been suggested to contribute functionally to the angiogenic cascade. β_1 integrin receptors may contribute to a variety of adhesive and migratory processes in all three phases of the angiogenic cascade. In addition, these integrins could also contribute to the regulation of protease expression, vacuole and lumen formation and differentiation, events thought to be associated with the latter phases of the angiogenic cascade.

membrane collagen type-IV, an ECM protein shown to be associated with mature blood vessels. Thus, ligation of type IV collagen may promote signals necessary for cellular morphogenesis and differentiation. In addition, it has also been shown that ligation of β_1 integrins can regulate the expression of proteolytic enzymes such as the matrix metalloproteinases (MMPs) [54]. Recently, it was shown that interaction of integrin $\alpha_5\beta_1$ with the 120FN cell-binding domain of fibronectin upregulated the expression of interstitial collagenase (MMP-1), stromolysin-1 and the 92 kDa gelatinase (MMP-9) [54]. In contrast, ligation of the CS-1 region of fibronectin by integrin $\alpha_4\beta_1$ suppressed this induction [54]. Thus, distinct integrins recognising different domains of the same molecule may transmit signals that differentially regulate matrix degrading proteases. Since it has been suggested that fibronectin is a major component of the provisional vascular matrix, it is possible that ligation of $\alpha_5\beta_1$ integrin expressed in angiogenic endothelial cells may regulate matrix degrading proteases thereby potentiating the angiogenic cascade. In this regard, recent studies have shown that specific antagonists of MMPs can inhibit capillary tube formation *in vitro* and angiogenesis *in vivo*, implying an important role for matrix degrading proteases in angiogenesis [16, 55]. Finally, it is also known that specific β_1 integrins can recognise the ECM protein laminin. In fact, numerous studies have implicated laminin in the regulation of cellular differentiation [46, 47, 56]. Therefore, ligation of laminin by endothelial cell β_1 integrins may potentiate biochemical signals necessary for endothelial cell differentiation.

Thus, the interaction between various β_1 integrins and specific ECM components may regulate a variety of cellular events during the angiogenic cascade (Figure 2). However, further studies on the functional roles of β_1 integrins in angiogenesis are needed to clarify these complex possibilities.

$\alpha_v\beta_3$ AND $\alpha_v\beta_5$ INTEGRINS IN ANGIOGENESIS

In addition to β_1 integrins, the vitronectin receptor $\alpha v\beta 3$ has also been implicated in the process of angiogenesis. Nicosia and colleagues utilised synthetic RGD-containing peptides to study the role of these adhesive sequences in the growth of microvessels from rat aorta [57]. These RGD peptides which disrupt both β_1– and β_3–ligand interactions inhibited microvessel outgrowth from rings of rat aorta embedded in collagen gels. Furthermore, Davis and associates used polyclonal antibodies directed to integrin $\alpha_v\beta_3$ or monoclonal antibodies to α_v, β_3 and β_1 integrins to block *in vitro* endothelial cord formation within matrigel cultures [44]. In contrast, Gamble and associates showed that antibodies to either integrin $\alpha_v\beta_3$ or $\alpha_2\beta_1$ actually enhanced *in vitro* tube formation within fibrin and collagen gels, respectively [58]. These apparently contradictory findings may be explained by the different assay systems and/or the concentrations of purified proteins used. For example, Ingber and colleagues demonstrated that the concentration of ECM ligands is critical in capillary tube formation *in vitro* [59]. Furthermore, since capillary tube formation probably requires cell migration, and in turn cell migration requires the cyclic formation and dissolution of cell adhesive contacts, it is possible that suboptimal concentrations of integrin antagonists may enhance migration by partially blocking cell adhesion. This partial loss of cell–ECM contacts may thus enhance capillary tube formation. In addition, it is well known that ligation of specific ECM components can influence cellular behaviour in different ways. Ligation of purified fibrin, for example, may lead to biochemical signalling events that are quite different from those that occur in the complex micro-environment *in vivo*. In fact, it has been shown that ligation of integrin $\alpha_v\beta_3$ by different ligands can lead to distinct cellular and biochemical responses [60].

To begin to study the role of integrin $\alpha_v\beta_3$ in angiogenesis *in vivo*, we showed that integrin $\alpha_v\beta_3$ is upregulated on both chick and human blood vessels after stimulation with either purified cytokines or solid human tumours [40, 41]. Furthermore, antagonists of $\alpha_v\beta_3$, including cyclic RGD peptides and antibodies, blocked angiogenesis induced by bFGF, TNF-α and a variety of solid human tumours [41]. We have recently extended these studies and shown that antagonists of $\alpha_v\beta_3$ can block tumour-induced human angiogenesis as well as the invasive behaviour of human breast carcinoma cells within the micro-environment of full-thickness human skin [61]. Importantly, these findings provide evidence suggesting that antagonists of $\alpha_v\beta_3$ may prove to be effective in treating a variety of human angiogenic diseases.

Further support for a role of $\alpha_v\beta_3$ in angiogenesis comes from recent studies by Hammes and colleagues [62]. Administration of cyclic peptide antagonists of αv integrins significantly inhibited retinal neovascularisation in a murine model of hypoxia-induced retinal angiogenesis with no apparent side-effects [62]. In recent studies by Friedlander and associates, antagonists of $\alpha_v\beta_3$ also inhibited angiogenesis induced by recombinant bFGF in the rabbit corneal model [63]. However, $\alpha_v\beta_3$ antagonists show little if any effect on VEGF-induced angiogenesis. In contrast, antagonists of the related vitronectin receptor $\alpha_v\beta_5$ significantly inhibited this response [63]. These surprising results indicate that not only can integrin $\alpha_v\beta_3$ play a significant role in angiogenesis, but depending on the specific cytokine used, the vitronectin receptor $\alpha_v\beta_5$ can also play a role. These findings suggest that there may be at least two distinct pathways leading to angiogenesis that can be defined, in part, by their dependency on a particular αv integrin. However, further studies on the relationship between $\alpha_v\beta_5$ and cyto-

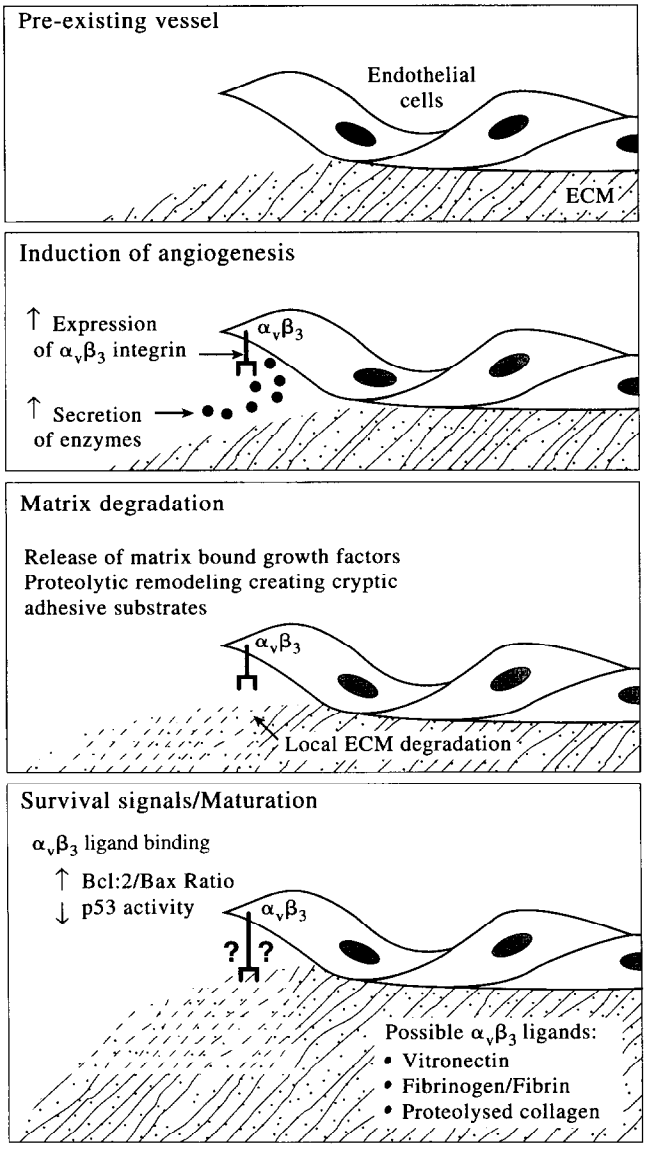

Figure 3. Model of $\alpha_v\beta_3$ function in angiogenesis. Pre-existing mature vessel: mature blood vessels are surrounded by a complex extracellular matrix (ECM). Induction of angiogenesis: stimulation of angiogenesis by a number of physiological mediators can lead to increased expression of integrin $\alpha_v\beta_3$ as well as increased release of a variety of matrix degrading enzymes including serine proteases and members of the matrix metalloproteinase family. Matrix degradation: proteolytic remodelling of the immediate vascular micro-environment may lead to the release of matrix-bound growth factors and possibly expose cryptic RGD adhesive substrates with which $\alpha_v\beta_3$ can interact. Survival signals/maturation: interaction of vascular integrin $\alpha_v\beta_3$ with intact ECM proteins or proteolytically modified ECM components such as collagen may suppress p53 activity, induce expression of Bcl-2 and downregulate Bax leading to an increased Bcl-2/Bax ratio. These $\alpha_v\beta_3$ mediated events could thus promote endothelial cell survival and thereby facilitate angiogenesis.

kine signalling will be necessary to establish the contribution of this integrin to the complex process of tumour angiogenesis. Finally, Drake and colleagues used a MAb-directed to integrin $\alpha_v\beta_3$ to disrupt precapillary lumen development in quail embryos. These findings demonstrate the critical importance of this integrin in normal vascular development [64]. Taken together, these data indicate that $\alpha_v\beta_3$ plays a critical role in angiogenesis in a variety of systems, including avian, rabbit, murine and human models.

MODEL OF $\alpha_v\beta_3$ FUNCTION IN ANGIOGENESIS

To begin to elucidate the possible mechanisms by which $\alpha_v\beta_3$ potentiates angiogenesis, we combined the use of both *in vitro* and *in vivo* models to study this question. Elegant studies by Montgomery and colleagues showed that ligation of proteolysed collagen by $\alpha_v\beta_3$ was of critical importance in the survival of melanoma cells in three-dimensional collagen gels [30]. These findings combined with others suggested the possibility that ligation of $\alpha_v\beta_3$ might also be important in endothelial cell survival. In fact, we recently demonstrated that antagonists of $\alpha_v\beta_3$ selectively induce apoptosis within angiogenic blood vessels *in vivo* [41]. Furthermore, recent studies by Stromblad and associates showed that systemic administration of antagonists of $\alpha_v\beta_3$ selectively activated p53 in endothelial cells and increased the p53-inducible cell cycle inhibitor, $p21^{WAF1/CIP1}$ [65]. Conversely, solid-phase ligation of $\alpha_v\beta_3$ expressed on endothelial cells suppressed p53 activity, blocked $p21^{WAF1/CIP1}$ expression and increased the bcl-2/bax ratio [65]. Interestingly, it has been suggested that both *BCL-2* and *BAX* genes have p53 responsive elements [66]. Moreover, it has also been suggested that a high bcl-2/bax ratio can promote cell survival [65, 66]. Thus, it is conceivable that ligation of $\alpha_v\beta_3$ provides a critical survival signal necessary for the growth and maturation of blood vessels during the angiogenic cascade (Figure 3). Since proteolytic remodelling of the vascular micro-environment is thought to occur during angiogenesis, it is possible that $\alpha_v\beta_3$ may interact with proteolysed or denatured collagen [30, 67]. In addition, other potential ECM components that may interact with $\alpha_v\beta_3$ include vitronectin, fibronectin, thrombospondin and fibrinogen/fibrin. However, which of these specific ECM proteins interact with $\alpha_v\beta_3$ during angiogenesis *in vivo* is not clear. Therefore, studies are currently underway to define physiologically important ligands for $\alpha_v\beta_3$ that could promote angiogenesis *in vivo*.

CONCLUSION

The integrin class of cell adhesion receptors have been shown to mediate a variety of physiologically important cellular events including adhesion, migration, invasion and cellular proliferation and survival. These cellular processes are thought to play fundamentally important roles in invasive biological events such as angiogenesis and tumour growth and metastasis. In this regard, solid tumours not only have the capacity to stimulate new blood vessel development, but in fact require extensive neovascularisation for their continued expansion. Therefore, identifying specific molecules that regulate angiogenesis and in turn understanding how these molecules may function during angiogenesis is of paramount importance. It is becoming increasingly clear that a number of integrin receptors, including members of the β1 subfamily and the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$, may play a fundamental role in angiogenesis. Therefore, further studies on the role of integrins in angiogenesis will undoubtedly lead to a more complete understanding of the complex role of cell adhesion molecules in invasive cell behaviour.

These studies in turn may lead to the development of novel strategies for the treatment of neovascular diseases.

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