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# Roles of molecules involved in epithelial/mesenchymal transition during angiogenesis

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## 1.ABSTRACT

Formation of vessels requires "epithelial-mesenchymal" transition of endothelial cells, with several modifications at the level of endothelial cell plasma membranes. These processes are associated with redistribution of cell-cell and cell-substrate adhesion molecules, cross talk between external ECM and internal cytoskeleton through focal adhesion molecules and the expression of several proteolytic enzymes, including matrix metalloproteases and serine proteases. These enzymes with their degradative action on ECM components, generate molecules acting as activators and/or inhibitors of angiogenesis. The purpose of this review is to provide an overview of the molecules involved in epithelial-mesenchymal transaction, including: the ECM, the cadherins, the integrins, the focal adhesion molecules, and the proteolytic enzymes. The initial clinical trials using physiological, synthetic and immunologic inhibitors against the described molecules for cancer treatment did not show the expected efficacy, in terms of reducing tumor progression. This is due to the fact that these molecules have multiple roles both in angiogenesis and tumor progression. Therefore, developing a strategy against induced angiogenesis requires an overview of all actors which are involved in this phenomenon.

## 2. INTRODUCTION

Growth of new blood vessels (angiogenesis) plays a key role in several physiological processes, such as vascular remodeling during embryogenesis and wound healing tissue repair in the adult; as well as pathological processes, including rheumatoid arthritis, diabetic retinopathy, psoriasis, hemangiomas, and cancer (1). Vessel formation entails the "epithelial-mesenchymal" transition of endothelial cells (ECs) "*in vivo*"; a similar phenotypic exchange can be induced "*in vitro*" by growing ECs to low cell density, or in "wound healing" experiments or perturbing cell adhesion and associated molecule functions.

The early process involves matrix remodeling, cell migration and invasion trough connective tissue, in proximity to the vessel wall. During this process several modification occurs at the level of EC plasma membranes, regarding the redistribution of cell-cell and cell-substrate adhesion molecules (2), the cross talk between external ECM and internal cytoskeleton through focal adhesion (FA) molecules and the increased expression of several proteolytic enzymes, including matrix metalloproteases (MMPs) (3,4), and serine proteases (5-7) (Figure 1).

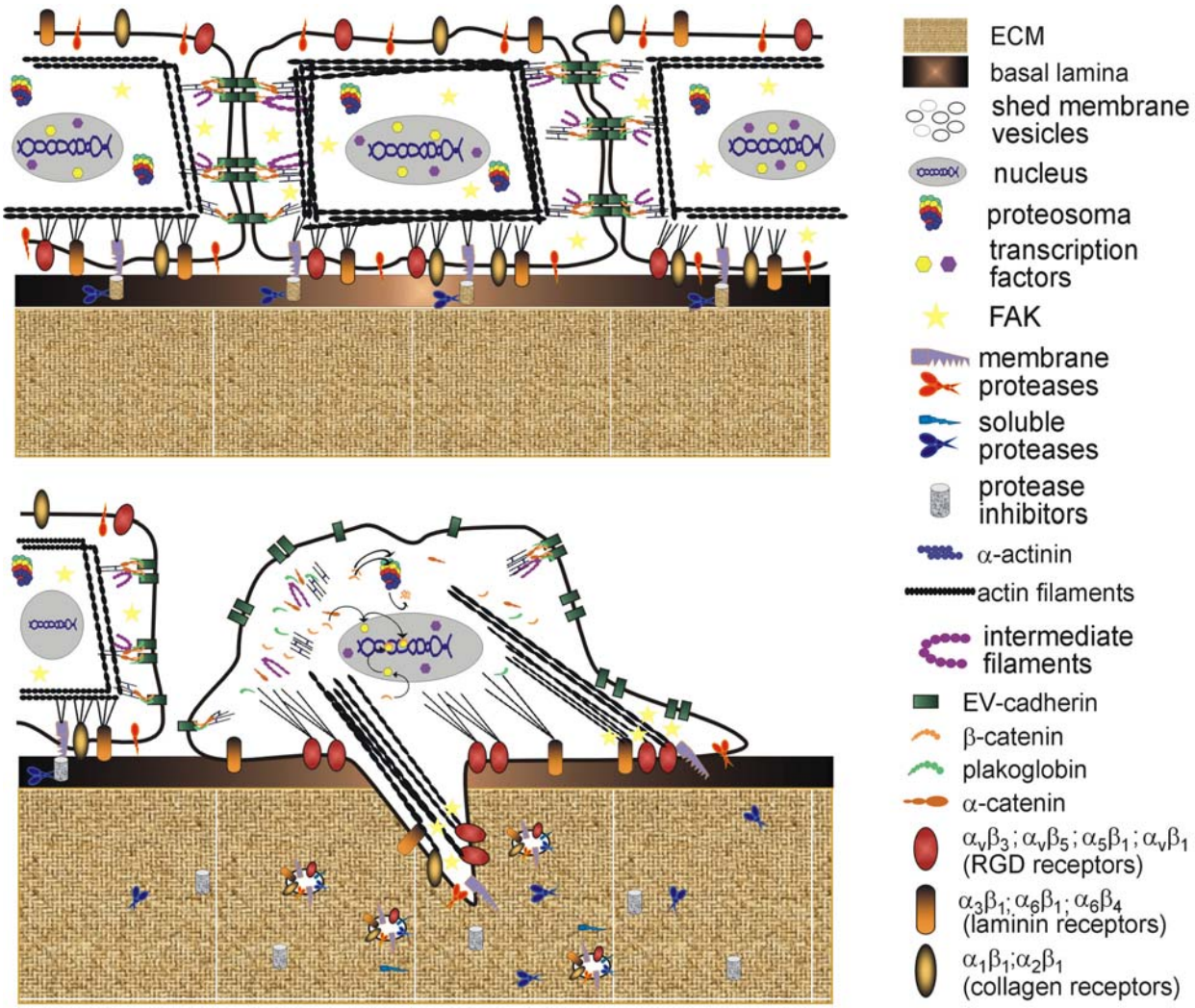


**Figure 1.** Schematic illustration of exchanges occurring during epithelial/mesenchymal transaction of endothelial cells. A - In endothelia cells forming well differentiated vessel specific cell-cell contacts, trough cadherin and other adhesion systems, and cell-substrate interaction, trough different classes of integrins, are established. The major cadherin class involved in this process is VE-cadherin, that stabilized through catenins stable contacts with actin and intermediate filaments cytoskeleton; another important cadherin in vessel differentiation is N-cadherin (not shown in the figure) this establish specific interaction with mural cells. Also stable interactions are establish between endothelial cell plasma membranes and basal lamina components trough integrins; the more representative integrins involved are those recognizing laminins, but also those having function of RGD receptors are present, less integrins with specificity for interstitial collagens. Also integrins, with their cytoplasm tails, take specific contacts through paxillin, vinculin, talin, alpha -actinin, etc., with actin cytoskeleton. Intracellular focal adhesion molecules are involved as molecular bridges between the intracellular and extracellular spaces that join together a multiplicity of environmental signals and mediate 2-way cross talk between the extracellular matrix and the cytoskeleton. On endothelial cell surface are present, also, proteolytic enzymes in inactive form. B - When endothelial cells acquire an invasive/migratory phenotype several exchanges in distribution and organization of molecules occurred. Cell morphology changes and specialized plasma membrane protrusions/structures are generated (invadopodia, philopodia, pseudopodia, etc.) and some released (shed membrane vesicles). The interactions mediated by EV-cadherin are missing and catenins are released in cytoplasm, in particular beta catenin can have two distinct destines: can be directed, after phosphorilation, to the proteosome system where will be degraded, or traslocated to the nucleus where, associated to LEF/TCF system, can work as transcription factor. Integrins are redistributed/reorganized and take new contacts with ECM, trough focal adhesion molecules, and in particular focal adhesion kinases, send new signals to cytoskeleton organization. On the other hand, activation of plasma membrane associated and soluble proteolytic enzymes occur; these generate in a time dependent way different proteolytic peptides that are involved in invasion (early steps) and differentiation (late steps) of new vessels.

The exact mechanisms used by ECM to support EC functions are complex and involve both plasma membrane peripheral structural exchanges as well as several regulative signaling pathways inside the cell, including the signaling pathways that control apoptosis, proliferation, the cytoskeleton, and cell shape. Moreover, the multiplicity of ECM components in the EC microenvironment and the variety of mechanisms needed to correctly organize the synthesis and degradation of ECM suggest an intricate level of complication for ECM to exert important and exact control on neovascularization and blood vessel maturation. Different ECM components, several generated by proteolytic activities present at the level of the EC invasion wall, act on the redistribution and functions of cell adhesion molecules. In the past two decades the varied roles of cell adhesion molecules in the development of new blood vessels have received widespread attention, focusing in particular on two families of adhesion molecules: cadherins and integrins.

Focal adhesions are molecular bridges between the intracellular and extracellular spaces that integrate a variety of environmental stimuli and mediate 2-way cross talk between the extracellular matrix and the cytoskeleton. Focal adhesion components are targets for biochemical and mechanical stimuli that evoke crucial developmental and injury response mechanisms including cell growth, movement, and differentiation, and tailoring extracellular microenvironment. Focal adhesions provide the vascular wall constituents with flexible and specific tools to exchange cues in a complex system.

The purpose of this review is to provide an overview of the molecules involved in epithelial-mesenchymal transaction of endothelial cells during angiogenesis, the subjects are: extracellular matrix (ECM), cadherins, integrins, focal adhesion molecules, and proteolytic enzymes.

## 3. EXTRACELLULAR MATRIX

There is extensive evidence regarding the role played by ECM to drive capillary morphogenesis through sustained signaling, resulting in persistent EC cytoskeletal reorganization and changes in cell form. A direct interaction between ECs and ECM is necessary during angiogenesis, especially during the sprouting of new blood vessels from the existing vasculature (8). Many "*in vitro*" experiments have shown the role played by the interstitial and provisional ECM components, including interstitial fibrin and collagen I, to exert a chemo tactic effect on sprouting ECs (9,10). Interstitial collagen is highly efficient at promoting haptotactic ECs migration *in vitro* (9). The sprouting of ECs is induced by both chemotactic gradients of angiogenic cytokine and haptotactic gradients of ECM; however, EC anchorage to ECM through integrins is essential for efficient MAPK activation by cytokines (11,12).

During angiogenesis, proliferating and migrating ECs arrange to form new 3D vessel networks. This phenomenon has been investigated widely during development, showing that the early stage of capillary morphogenesis involves transition of endothelial precursor cells to a spindle-shaped morphology (13). Contemporaneous with this morphology change, EC precursors align and connect into solid, multicellular, precapillary cord-like structures that form an integrated multilateral network (14,15).

During angiogenesis, the ECM act as a 3D network in which individual and clustered ECs transducers mechanical signals to other ECs, also at a significant distance. In this way, through mechanical forces occurring inside to ECM, ECs are able to create specific guidance forces to form interconnected cords. This kind of organization provides an instrument for ECs to organize into multicellular structures without the initial requirement of cell-cell contact (15,16).

It was definitively demonstrated that 3D interstitial collagen type I induces ECs in culture to undergo marked morphology changes that closely imitate the precapillary cord formation observed during embryonic vasculogenesis and adult angiogenesis. Moreover, the highly specific angiogenic cytokine VEGF, which induces sprouting angiogenesis also induces micro vascular ECs to express integrins alpha 6 beta 1 and alpha 2 beta 1(17), which are the highly specific interstitial collagen receptors on micro vascular ECs. Furthermore, antagonism of these integrins inhibits dermal and tumor angiogenesis "*in vivo*" (9). Experiments performed using proline analogues, that interfere with collagen's triple helix assembly, and beta-aminopropionitrile, which inhibits collagen cross-linking, have shown a reduction in both of neovascularization in animal models, indicating that collagens play a crucial role in angiogenesis (18).

The mechanisms used by collagen I to induce EC formations of cord-like structures have begun to be identified. The collagen I-interaction with integrins alpha 6 beta 1 and alpha 2 beta 1in micro vascular ECs *in vitro* has been shown to suppresses cAMP and consequently block the activity of cAMP-dependent protein kinase A (PKA). Blocking PKA action induces actin polymerization that contributes to the formation of stress fibers and EC contractility (19). Moreover, collagen I stimulation of micro vascular ECs "*in vitro*" induces activation of Src kinase and the GTPase Rho, also through interaction with beta 1 integrins (20). The suppression of PKA activity, along with activation of Src and Rho, is a key mechanism through which collagen I induces capillary morphogenesis of micro vascular ECs.

It is interesting how collagen I and laminin-1 signaling in micro vascular ECs regulate different stages of angiogenesis. In early stages of angiogenesis, during the sprouting and proliferative stages, the laminin-rich basal lamina is degraded; consequentially a reduction of EC–laminin interactions occurs. The reduction of laminin substratum results in a minor Rac activity and consequently a loss of Rac function in supporting the integrity of cell-cell junctions during this phase. Moreover, as previously described, sprouting ECs interact with interstitial collagens and invade it, resulting in activation of Src and Rho,

suppression of PKA, and initiation of capillary morphogenesis. Then, as the newly formed capillary sprouts mature into new vessels with mature lumens, the intact basement membrane is synthesized. Newly formed basal lamina sequesters ECs from interstitial collagens and thereby reestablishes normal activation levels for Rac, Rho, Src, and PKA. Thus, the laminin-rich basement membrane is involved not only in maintaining the integrity of the mature endothelium but also requiting and thereby insulating ECs from interstitial collagens. On the contrary, degradation of basement membrane exposes ECs to interstitial collagens and activates all signaling pathways that induce cytoskeletal reorganization and sprouting morphogenesis.

### 3.1. ECM and integrins

Some data indicates that EC integrin interactions with interstitial matrix proteins (i.e., interstitial collagens and fibrin/fibronectin) are key receptors in stimulating EC tubular morphogenesis as well as being involved in EC activation (19-22). The most important integrins responsible for these interactions are alpha 2 beta 1, alpha 6 beta 1, and alpha v beta 3, alpha 5 beta 1which are collagen and fibrin/fibronectin receptors, respectively (9,16,21,23-25). In a different way, basement membrane laminin-binding integrins such as alpha 6 beta 1and alpha 3 beta 1 are important in the process of tube stabilization.

Integrin alpha 6 beta 1 has been reported to control cord formation in bi-dimensional angiogenesis systems in which ECs are placed on the surface of basement membrane matrix gels (14,26,27). Therefore, it is essential to consider that several integrins may act together in regulating vascular morphogenesis (i.e., alpha 2 beta 1and alpha 6 beta 1 in collagen matrices or alpha 5 beta 1or alpha v beta 3in fibrin matrices) (21,23) and vascular stabilization (possibly alpha 6 beta 1or alpha 3 beta 1 in combination with alpha 2 beta 1or alpha 6 beta 1) and in this way generate a possible combinations of structural signals.

Several experiments performed in 3D collagen matrices has shown a close relationship between integrins and pinocytic intracellular vacuoles to form lumen during capillary morphogenesis (21) (16,24,28). Intracellular vacuole and lumen formation requires the activity of MT-MMPs on the EC surface. A great deal of data strongly suggest that MT-MMPs are the critical regulators of EC invasion into 3D collagen or fibrin matrices (23,29-37). On the other hand, many soluble MMPs induced in expression during EC tubular morphogenesis have not been found to be involved in ECs invasive/morphogenic events (23,32,38).

### 3.2. Basal lamina components

The primary component of basement membrane are the laminins; the other basement membrane components such as collagen type IV variants, perlecan (basement membrane heparan sulfate proteoglycan-2), nidogens, and collagen type XVIII are accessory components (39-42). EC basement membranes are primarily composed of the laminin-8 (43,44) and laminin-10 isoforms (44-46), and of smaller amounts of laminin-9 (alpha 4 beta 2 gamma1) or laminin-11 (alpha 5 beta 2 gamma 1). However, EC basement membranes principally contain the collagen type IV isoform (alpha 1)2 beta 1 (47). Moreover, early work indicated that fibronectin may provide the temporary matrix necessary to vascular morphogenesis, and laminins are synthesized later for stabilization (48); more recent work suggests the involvement of laminin-8 and collagen type IV to provide a provisional ECM scaffold during early tubular morphogenesis, substituted by laminin-10 in mature basement membrane (39) (43).

## 4. CADHERINS

The cadherin family is made up of calcium-dependent cell adhesion molecules responsible for cell-to-cell recognition and adhesion in solid tissues (49,50). They are expressed in several types of tissues with some specificity: E-cadherin is typically present in epithelial cells, N-cadherin in the nervous system, smooth muscle cells, fibroblasts and endothelial cells, VE-cadherin is specific to the endothelium (49,51). Classic cadherins interact through their cytoplasmic tail with beta-catenin and plakoglobin/gamma-catenin, which in turn bind to alpha-catenin. Through catenins, cadherins interact with the actin cytoskeleton to stabilize the junctional structure and contribute to maintening cell morphology and control of cell motility.

Cadherins typically include a signal sequence and a propeptide. The cadherin extracellular domain is divided into cadherin repeats, which are numbered from the N terminus. Type I and Type II cadherins typically have 5 extracellular repeats, a juxtamembrane domain that binds p120 catenin, and a catenin-binding domain that interacts with beta-catenin. T-cadherin (also called H-cadherin) does not have a transmembrane domain but is linked to the membrane by a lipid tail. LI cadherin is unusual in that it has two inserts in the extracellular domain that disrupt the cadherin repeats. Desmoglein 1 is unusual because it has only 4 extracellular repeats; desmogleins 2 and 3 each have 5 extracellular repeats. Desmocollin is unusual because it is alternatively spliced in the cytoplasmic domain to produce type a and type b splice variants. CNR protocadherins are unique because they have a binding site for Fyn in the cytoplasmic domain. FAT is an unusual cadherin because it has 34 extracellular repeats. Flamingo is an unusual cadherin because it crosses the plasma membrane seven times and likely binds G proteins. The Ret proto oncoprotein is a member of the cadherin superfamily because it has 2 cadherin repeats in its extracellular domain (52).

Cadherins mediate several intracellular signals through specific intracellular partners as kinases and/or phosphatases that could be associated with cadherin/catenin complexes. Endothelial cells express different cadherins, which are involved in transmiting signals to exert distinct functional roles. VE-cadherin is a specific and major constituent of adherent's junctions in ECs. This molecule has several roles enclosing to protect ECs from apoptosis

and contributing to contact inhibition of endothelial cell growth. Another high express member of the family is the N-cadherin and its important role is to modulate VE-cadherin expression. T cadherin, R-cadherin and VE-cadherin 2 were also detected in ECs, but their role in vascular development or angiogenesis is still unclear. On the other hand, members of the desmocollin or demoglein cadherin family, which generally are associated with desmosomes in other cell types, are not present in ECs. Therefore, their intracellular partners such as plakoglobin or desmoplakin are free to associate with VE-cadherin and probably exert a different functional activity.

### 4.1. Cadherins in angiogenesis

Knocking out the gene codifying VE-cadherin was the first evidence of its involvement in angiogenesis; in fact doing so induced an early lethal phenotype in the mouse (53). A question instinctively arises: is VE-cadherin inhibition instrumental to inhibiting angiogenesis in the adult ? Some monoclonal antibodies (mAbs), recognizing the extracellular domain EC-1 of VE-cadherin, were able to prevent junction assemblage and to provoke disassembly of preexisting ones (54). Low doses of these mAbs inhibited angiogenesis in different experimental tumors in the mouse (55). On the other hand, at higher concentrations of the mAbs, vascular permeability increased, both in the lung and heart, thus inducing toxic effects (56). Different roles are played by VE-cadherin in angiogenesis regulation. In addition, if adhesive properties of VE-cadherin are also essential to stabilize endothelial cell-cell adhesion, junctional adhesive proteins are present in high numbers in endothelial cells (57); this assumes a high degree of redundancy. Thus, the dramatic effects observed by inactivation of VE-cadherin expression or function strongly suggest that they are due to intracellular signaling. Previous work showed that VE-cadherin associates with VEGF-R2 and modulates its signaling pathways. When VE-cadherin is silenced, VEGF-R2 is heavily tyrosine phosphorylated and in this condition activate uncontrolled endothelial cell growth; this phenomenon is accompanied by an increase in cell apoptosis (58). Another way used by VE-cadherin in signaling is by sequestering beta-catenin at the plasma membrane level; when beta catenin is detached by the cytoplasmic domain of VE-cadherin, it can translocate to the nucleus and act as a transcription factor. Tyrosine phosphatases have been shown to be involved in promoting cadherin-mediated adhesion, likely by dephosphorylating VE-cadherin and catenins (58,59). Phosphorylation in tyrosine of beta-catenin may be an important mechanism of regulation. Phosphorylated beta catenin is less tightly bound to VE-cadherin and this in turn may lead to a decrease in anchorage of the complex to actin (60).

Recently data points to a regulatory mechanism of VE-cadherin turnover at the membrane level, which involves p120ctn (61). This protein acts as a controller of the amount of classic cadherins at plasma membrane level: it binds to a juxta-membrane region of the cytoplasmic cadherin tail and inhibits endocytosis. Modifications of p120ctn expression or inhibition of its binding to the cadherin tail may induce cadherin internalization and degradation. When associated in the cadherin/catenin complex, p120ctn may also control several other activities that include adhesion, cadherin clustering and cell signaling (62).

It was also observed that small GTPases play a role in regulating cadherin and in particular VE-cadherin adhesive functions. For example, Rap-1 was found to regulate the adhesive properties of E-cadherin and VE-cadherin, reducing endothelial permeability and leukocyte diapedesis through endothelial cell junctions (63) (64). Other data has shown the involvement of GTPases in stabilizing or destabilizing endothelial VE-cadherin, functioning in culture conditions, cellular context and put forward stimulus (51).

The other abundant cadherin expressed in the endothelial cell is the N-cadherin (65); however, its role and functions, including angiogenesis, has remained largely elusive. Recent observations showed a major role played by N-cadherin in the interaction of endothelial cells with mural cells (pericytes and vascular smooth muscle cells), a process that is fundamental to the maturation and stabilization of the vasculature (66,67). N-cadherin is clustered at the contact sites between endothelial and mural cells in different tissues (68,69); for example, neutralizing it during chicken brain development resulted in defective pericyte adhesion to the brain vessels, leading to an aberrant vascular morphogenesis and hemorrhages (70).

## 5. INTEGRINS

Several members belong to the integrin family; they are heterodimeric transmembrane glycoproteins and are generally involved in cell-extracellular matrix and sometimes in cell-cell adhesion interactions. They consist of a single alpha-subunit non-covalently interacting with a single beta-subunit. In mammals, there are 18 types of beta-subunits and 8 different types of alpha-subunits that can interact to form almost 24 known different integrins; the heterodimer conformation conferred them ligand specificity (71,72).

ECs express several integrins including alpha 1beta 1, alpha 2 beta 1, alpha 3 beta 1, alpha 6 beta 1, alpha 6 beta 4, alpha 5 beta 1, alpha v beta 3 and alpha v beta 5, and these recognize several ligands (73). Alpha-1 beta 1 and alpha 2 beta 1, as just reported, are principally collagen receptors but can also interact with laminin. Alpha 3 beta 1, alpha 6 beta 1 and alpha 6 beta 4 principally recognize laminin receptors, but it has also been shown that alpha 3 beta 1 can interact with reelin and thrombospondin. Alpha 5 beta 1 and the alpha v-integrins, alpha v beta 3 and alpha v betas 5, all bind to extracellular matrix molecules via an arginine-glycine-asparagine (RGD)-binding site. The highly specific ligand for alpha 5 beta 1 is fibronectin, for alpha v beta 5 is vitronectin, while alpha v beta 3 bind several substrates including: vitronectin, fibronectin, von Willebrand factor, thrombospondin, osteopontin, laminin and denatured collagen.

Each integrin with its cytoplasmic tail makes connections to actin cytoskeleton with the exception of alpha 6 beta 4 which interacts with intermediate filaments.

Generally, in bi-dimensional cell culture systems, cell-substrate interacting integrins are expressed on the basal face of cells, and could assemble to form clusters known as focal contact sites. Integrins in focal contact sites form complexes in the cytoplasm portion with linker proteins (paxillin, vinculin, talin and alpha -actinin, etc.) that connect them to actin cytoskeleton (71,74-78). Many signaling molecules are also linked with focal contact sites, thus connecting integrin-mediated adhesion with signal transduction pathways that control various processes such as migration, proliferation and differentiation. These signaling molecules include focal adhesion kinase (FAK), members of the Src family of non-receptor tyrosine kinases and members of the Rho family of small GTPases.

Although integrin cytoplasmic tails are much smaller than their extracellular domains, they can have key roles in integrin signaling events; by separating, twisting, pistoning and hinging the tails, all considered mechanisms to allow activation. On the other hand, it is generally recognized that most integrins, including endothelial integrins, can have 'on' and 'off' states. Recent advances in deciphering affirm this conviction concerning the crystal structures of integrins, that associated with data from previous studies, have led to the conclusion that integrin activation coincides with changes in integrin conformation (79-82).

### 5.1 Integrins in angiogenesis

In blood vessels, alpha 6 and alpha 4 are constitutively expressed in capillary and in sprouting structures; alpha v beta 3, alpha v beta 5 and alpha 2 beta 1 are scarcely present in quiescent vessels but highly in sprouts (83), with alpha v beta 3 expression enhanced principally upon bFGF treatment (84). Alpha 1 beta 1, alpha 3 beta 1 and alpha 5 beta 1 are expressed at low levels in quiescent vessels but at least alpha 5 beta 1 is regulated up during angiogenesis (85). Beta 1-integrin is crucial in angiogenesis, in fact beta 1-integrin-null embryos die early in gestation and do not develop far enough to begin to produce vasculature (86,87). Moreover, beta 1-null embryoid bodies are resistant to vascular endothelial growth factor (VEGF)- induced proliferation and branching (88). VEGF stimulation of endothelial cells induced an increased regulation of expression in the collagen receptors alpha-1 beta 1 and alpha 2 beta 1; moreover, collagen I ligation of alpha-1 beta 1 and alpha 2 beta 1 is a decisive step in initiating cord formation of endothelial cells (19). Recently, a new inhibitor of alpha 2 beta 1, E7820, has been used to inhibit tumor angiogenesis (89-91), thus indicating important roles for these integrins in angiogenesis. A different role is played by alpha-1 beta 1 in pathological angiogenesis; in fact, alpha-1-integrin deficiency causes an increase in MMP7 and MMP9 production and these metalloproteinases can cleave circulating plasminogen to make angiostatin, a well known inhibitor of angiogenesis.

The alpha 3 beta 1 and alpha 6 beta 4 (laminin receptors) did not have the right attention relative to their roles in angiogenesis. See, also, that the genetic ablation of both alpha -subunits results in lethal phenotypes within hours after birth (92-94). On the other hand, alpha v beta 3 and alpha v beta 5 have received a great deal of attention in relation their roles in angiogenesis. Specific inhibitors, including a function blocking monoclonal antibody directed against the extracellular domain of alpha v beta 3 (LM609) and cyclic RGD peptides, were shown not only to block vitronectin binding of endothelial cells, but also to inhibit angiogenesis in tube formation assays *in vitro*, embryonic neovascularization (95), retinopathy of prematurity models (96,97), arthritis models (98) and in murine tumor models (99,100). Indeed antagonists of alpha v beta 3 such as the humanized version of LM609, Vitaxin, are presently undergoing clinical trials (101).

Several other ECM components having an RGD sequence, apart vitronectin and fibronectin, have pro-angiogenic effects interacting with alpha v beta 3. In particular, proteolytic cleaved collagen typeIV exposes a cryptic site that is normally hidden within its triple helical structure (102) or thrombin, a coagulation factor containing an RGD sequence, can up regulate the expression of several integrins enclosing alpha v beta 3 (103). Growth factor receptors, involved in angiogenesis, can also directly bind alpha v beta 3 via interactions, which occur in their extracellular domains. For example, VEGF-receptor 2 (Flk-1), PDGFb and insulin receptors can all be co-precipitated with alpha v beta 3-integrin, (104-106). It is clear that alpha v beta 3 can bind directly to several molecules, not necessarily in an RGD-dependent fashion, and that such interactions can enhance angiogenesis. On the other hand, proteolytic cleavage of matrix molecules produces fragments of ECM proteins that have alpha v beta 3-binding ability and anti-angiogenic properties. These include angiostatin, a fragment of plasminogen (107), and endostatin, a proteolytic fragment of collagen XVIII (108). Both these molecules can bind integrins alpha v beta 3 and alpha 5 beta 1 (109-112). Another molecule is the tumstatin; it is a proteolytic fragment of collagen type IV, containing the NCI domain of the collagen alpha 3 chains, and binds alpha v beta 3 in a non-RGD-dependent fashion (113,114). Tumstatin does not block vitronectin binding to alpha v beta 3 but does inhibit angiogenesis *in vivo* (115-117).

## 6. FOCAL ADHESION MOLECULES

Focal adhesions (FAs) are molecular bridges between the intracellular and extracellular spaces that join together a multiplicity of environmental signals and mediate 2-way cross talk between the extracellular matrix and the cytoskeleton. Focal adhesions provide vascular wall constituents with flexible and specific tools for exchanging cues in a complex system. Signal promulgation sees the controlled interaction and aggregation of more than 50 diverse adapted to specific environmental cues. FAs include a large number of examples of cellular signaling integration; as conformational changes occurring in integrin ligand binding domains and modular localization of intermolecular adapter interactions.

Specificity in integrin signaling is made possible by the particular alpha and beta chains that form the

heterodimeric pair and the distinct binding interactions of the cytoplasmic tails of these pairs with the actin cytoskeleton and with signaling molecules. Integrin signaling occurs via a large collection of intracellular second message systems including calcium channels, phosphatidylinositol-4,5-bisphosphate, phospholipase-C gamma, the Na/H antiporter, tyrosine and serine/threonine kinases, phosphatases, Rho family GTP-binding proteins, mitogen-activated protein (MAP) kinases, and cyclin D1 (118-125). The activation and spatial distribution of integrin-mediated cell-matrix adhesion sites on the cell surface can greatly influence different signaling proceedings including the directional control of actin organization by vinculin and Arp2/3, and vascular cell differentiation (126,127). The bi-directional characteristic of integrin signaling is based on mutual, intramolecular, allosteric changes that are initiated in either the cytoplasmic tail or the extracellular domain and then transmitted to the other end of the molecule. This permits ECM ligation to promulgate signals in an "outside-in" direction, whereas intracellular changes in cytoskeletal tension can cause "inside-out" signaling (119,128,129).

Mutual statement between integrins and proteins that regulate the actin cytoskeleton is an important characteristic of FA signaling. Thus, Rho activation accentuates FA growth (130), while integrin engagement has direct effects on Rho activity and, as a consequence, Rho-mediated FA revenue via Src21 and focal adhesion kinase (FAK) (131-134). Additionally, integrins control the availability and turnover of Rac by directing Rac connections with caveolae and the plasma membrane (135,136). Moreover, the Rho family of GTP-binding proteins and myosin light chain kinase (MLCK) generate dynamic inside-out integrin signals via cytoskeletal remodeling (137-141).

Membrane extension and cytoskeletal tension are also synchronized by FAK through several interactions with regulators and effectors of Rho family proteins, including the GTPase-activating proteins p190RhoGAP35 and GRAF (142), the Rac effector p95PKL (143) and the guanine nucleotide exchange factors ASAP1, Trio, and PIX (144-146). FAK also binds the adapter proteins paxillin and tyrosine phosphorylates at Y31 and Y118, by this means altering paxillin connections with the alpha 4 beta 1 integrin and the PKL-PIX-PAK complex at the leading edges of spreading lamellopodia (147). FAK influences cell motility and directional control by mediating FA interactions with caveolae and microtubules. Tyrosine-phosphorylated caveolin 1 and 2 co localize with activated (Y397-phosphorylated) FAK at the leading edges of migrating human micro vascular endothelial cells (148). In an apparent 2-step cooperative process with Src, FAK tyrosine phosphorylates endophilin A2 inactivates it and inhibits internalization of MT1-MMP. In this way, FAK increases the amount of this membrane-bound protease on the cell surface and aids tissue invasion (149).

While cell survival, mobility, and invasion may all be balancing programs, the amount and diversity of cellular processes and binding interactions in which FAK participates suggest that FAK serves as a cellular signaling "switch." Proposed mechanisms for this molecular switching include alternate folding of the FAK FA targeting domain, principal to graded accessibility of Y925, and/or individual degrees and patterns of tyrosine phosphorylation (150-152). However, additional investigations of the mechanisms that cause FAK switching between its many signaling and scaffolding roles are required.

## 7. PROTEOLYTIC ENZYMES

Proteolytic enzymes can be divided in four main classes: Aspartic-, Cysteine-, Matrix Metallo- and Serine-proteases. The enzymes belonging to the first two classes are commonly involved in apoptosis and cell proliferation processes, respectively, even if some exceptions subsist. Proteases belonging to the other two classes are prevalently implicated in cell migration/invasion processes, such as in ECM remodeling, and many of them are directly and/or indirectly involved in angiogenesis; these preferentially exert their function on extracellular matrix components also if these act-on other substrates as growth factors, cytokines, etc. These molecules are synthesized as soluble- and/or plasma membrane associated proteolytic enzymes. Soluble forms of MMPs and Serine-proteases are all released in inactive conformations and enzymatically activated extracellularly. A similar activation mechanism occurs in the plasma membrane-associated forms of MMPs, the MT-MMPs, which are also enzymatically activated. While a dissimilar mechanism of activation is adopted by several integral plasma membrane serine peptidases, they are synthesized in a monomeric inactive form that, on plasma membranes, associate to form proteolytically active dimers (153).

MMPs belong to a family of over twenty members of zinc-containing endopeptidase activities and have the capability to process several ECM components (154). The family members have been divided into different groups on the basis of their structure and/or digestive substrates. Structurally the MMPs are organized in different domains that change in number and composition between the members of the family. MMP1, MMP8 and MMP13 are collagenases, they degrade the native helix of types I, II, III, and other fibrillar collagens; structurally they are organized in molecules having signal peptide, the propeptide domain, the catalytic domain containing the zinc-binding site plus a simple hemopexin-like domain connected to the catalytic domain via a proline-rich hinge region, (155-158). MMP3, MMP10 and MMP11 are stromelysins, structurally very similar to the collagenases, but they have a broad spectrum substrate specificity and degrade several ECM components, such as proteoglycans, fibronectin, and laminin (155,159,160). Then, there is MMP7, the matrilysin, the simplest structural subclass of MMPs organized in a signal peptide, a propeptide domain, and the catalytic domain containing the zinc-binding site (155,161). MMP2 and MMP9 (gelatinases) are very different, they are comparable in structure to collagenases and stromelysins with an additional region composed of three fibronectin type II

repeats within their catalytic domains; their preferential substrates are the denatured collagens (gelatin), the types IV, V, VII, and X native collagens, fibronectin, and laminin (162). The MMPs described, together with MMP12, a metalloelastase (163), MMP26, matrylisin-2 (164), and MMP28, epilysin (165,166), are all soluble forms; however some of them are also located on the plasma membrane through direct interaction with specific connectors. Included here are 6 members MT-MMPs 1-6, which are connected to the cell surface via a C-terminal trans-membrane domain or glycosylphosphatidylinositol anchor (167-170). The MT-MMPs act on numerous diverse substrates including: gelatin, fibronectin, aggrecan and some other ECM substrates (155). The membrane type matrix metalloproteases are type I peptidases, their orientation on the plasma membrane is with the N-terminal domain in the extracellular compartment and C-terminal domain in the cytoplasm: included in this class are MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP. A different mechanism of interaction enzyme/cell plasma membrane via the glycosylphosphatidylinositol (GPI) tail is observed for MT4-MMP and MT6-MMP, which belong to type III peptidases.

There are more than 40 members with different roles in the serine-protease family; however, only a restricted number of them are involved in angiogenesis. They are well conserved within the human genome (171) and amongst species(172). They use a common chemical mechanism of proteic substrate hydrolysis, which is involved the catalytic triad composed of histidine, aspartate and serine amino acids. The members of the family are involved in several functions; digestive enzymes (173), blood coagulation and fibrinolytic enzymes(174), glandular kallikreins (175,176), granzymes (177), and Type-II Transmembrane Serine Proteases (TTSPs) (153,178). The greater part of the members of the family are soluble molecules, which are secreted in inactive form and than enzymatically activated. The individual serine proteases show an astonishingly broad mixture in their substrate specificities, and the proteases of the plasminogen activation system fall at either end of this field. Plasmin has a very small broad specificity and is a very efficient enzyme; its activity is analogous to trypsin, cleaving C-terminally Lys and Arg amino acids. On the other hand, uPA and tPA, activators of plasminogen, are tremendously precise and a very small number of substrates for these enzymes have been identified (179-181). Further targets for plasmin are other proteases, the MMPs; it has been shown activate to completely, or in part, interstitial collagenase (MMP1), stromelysin-1 (MMP3), matrilysin (MMP7), gelatinase-B (MMP9), metalloelastase (MMP12), and collagenase-3 (MMP13) "*in vitro*" (182) and, for some of them, activation has also been demonstrated "*in vivo*" (183). Moreover, plasmin is involved in mobilization of matrix-sequestered FGF-2 and it is an activator of the brain-derived neurotrophic factor (BDNF), of b-NGF, and of latent TGF-beta (184-186).

An emerging class of serine protease involved in angiogenesis is that of the TTSP family, in particular seprase and DPP4 appear to be directly implicated in new vessel formation (7); they are inducible, specific for proline containing peptides and macromolecules, and active on the cell surface. Both human DPP4 and seprase have a 68% identity in the catalytic region and a conserved serine protease motif G-X-S-X-G. Recent studies also showed that, like DPP4, other members of the peptidases family cleave prolyl peptide bonds (Pro-Xaa) (153). Unlike proteases, seprase and DPP4 together with other members of the family don't present a propeptide and are then not activated enzymatically; however their dimerization is required to exert prolylpeptidase and/or gelatinase activities. DPP4 appears to have prolyl exopeptidase activity; some of its natural substrates include neuropeptide Y (187), substance P (188)and beta chemokines such as eotaxin, SDF-1 (stromal derived factor) and RANTES (regulated on activation normal T cell-expressed and secreted) with either L-proline, L-hydroxyproline, or L-alanine at the penultimate position (189,190). Along with exopeptidase activity, DPP4 also exhibits endopeptidase activity towards denatured collagen (191). Since proteolytic activity of DPP4 was observed for several denatured collagens (type-I, -II, -III and –V) a contribute of DPP4 in collagen trimming and metabolism was suggested. Concerning seprase, studies of gelatin zymography have shown it to be a membrane gelatinase, and studies using a sensitive fluorogenic assay have also demonstrated its prolyl peptidase activity (192).

### 6.1. Proteolytic enzyme inhibitors

The functions of MMPs are controlled by endogenous inhibitors, those best characterized are the tissue inhibitors of metalloproteinases (TIMPs). Four members belong to this group, TIMP-1 to -4, which reversibly inhibit MMPs in a 1:1 stoichiometric fashion (193). Their inhibitory function is mediated by the N-terminal domain (194,195). Peptides and antibody-blocking experiments (196,197) together with NMR (198) and X-ray crystallographic (199,200) and mutational analyses (201) (202) have confirmed direct contact between TIMPs N-terminal region and the catalytic site of MMPs. TIMPs have different specificity: for example, TIMP-2 and TIMP-3 are MT1-MMP inhibitors but not TIMP-1 (203); the last one is a stronger inhibitor for MMP9 and a poor inhibitor of MT3-MMP. TIMPs also exert their inhibitory effects on several members of ADAM family. Moreover the TIMPs are involved in independently promoting cell growth via their proteolytic enzyme inhibitory activity (204) (205). TIMPs are not the only endogenous MMP inhibitors; the alpha 2-macroglobulin, an abundant component of plasma, represents the most important inhibitor of MMPs present in tissue fluids (206). Another inhibitor of MMPs is RECK (REversion-inducing Cysteine-rich protein with Kazal motifs), a glycosylphosphatidylinositol membrane-anchored glycoprotein generally expressed in human tissues (207); whose inhibitor activity is prevalently directed at MMP2 and MT1-MMP. Other MMPs inhibitors, structurally similar to TIMPs, are the proteolytic processing procollagen C-terminal proteinase enhancer protein (PCPE), which releases a C-terminal inhibitory fragment (208) and the noncollagenous NC1 domain of type-IV collagen (209).

The inhibitors of serine proteases, and of the plasminogen activation system, commonly belong to the serpin (serine protease inhibitor) super family. Members of the family have a conserved structure and use a metastable conformation to exert their inhibitory activity. They exert their inhibitory role by initially forming a non-covalent Michaelis-type complex leadsing to cleavage of the scissile bond (Arg-Met) and the formation of a covalent connection between the reactive serine of the protease and the backbone carbonyl of serpin in the reactive center loop; this is followed by a conformational restructuring that leads to enzyme inactivation (210). The major serpin inhibitor of uPA and tPA is the plasminogen activator inhibitor-1 (PAI-1); for both plasminogen activators PAI-1 is an extremely competent inhibitor with a $K_M$ 2 x $10^7$ $M^{-1}$ $s^{-1}$ and it is expressed in several cell types in highly regulated manner, although low concentration (less than 1 nM) is found in circulation. On the other hand, PAI-2 has a small limited expression pattern, prevalently in monocytes and in placenta; it lacks signal peptide and seems to exert its role intracellularly. Moreover, it is a very poor inhibitor of tPA (5 x $10^3$ $M^{-1}$ $s^{-1}$ for single-chain tPA, less than $10^3$ times PAI-1) and exerts modest action on uPA (2 x $10^6$ $M^{-1}$ $s^{-1}$) (211). Other serpins are less active than PAI-1, but are not thoughy to have very important functional roles. The plasmin is regulated by alpha 2-antiplasmin; this serpin is extracted in the liver and in the plasma (1 micro molar) and it is probably the most efficient member of serpin family, with a constant association of about $10^8$ $M^{-1}$ $s^{-1}$ (212,213).

Several TTSPs are inhibited by serpin molecules such as aprotinin, alpha 2-antiplasmin and PAI-1. Recently, it has been demonstrated that potent inhibitors for furin and kexin, belonging to the convertases family, could be created by the introduction of their recognition sequences within the reactive site of the proteolytic enzyme inhibitor eglin c (214,215). Three-dimensional studies on eglin c structure have appeared to generate highly efficient inhibitors for matripstase (216), and to infer the possibility of generating other inhibitors against additional members of TTSP family.

### 7.2. Proteolytic enzyme in angiogenesis

Several MMPs are implicated in angiogenesis, but only severe defects in angiogenesis have been observed in MT1-MMP knockout mice; for many others no apparent effects were observed (217) (218). In addition a number of serine proteases are involved in angiogenesis, but knocking out several of them did not cause the expected effects; for example, the knockout of the gene codifying plasmin and urokinase has not shown any effects (219). However, the knockout of DPP4 and other membrane associated serine proteases results in damage to the angiogenesis (220). Both MMPs and serine proteases have positive and negative effects on angiogenesis regulation; several of them are involved in vascular development and in vascular morphogenesis (221,222).

In physiological angiogenesis there is a well controlled equilibrium between angiogenic factor signaling; exogenous and endogenous angiogenetic activators and inhibitors are present in proper balance. On the other hand, in pathological angiogenesis, the equilibrium becomes perturbed. MMPs are essential for endothelial cell migration/invasion and tube formation (223). Some MMPs are implicated in endothelial cell migration and invasion of fibrin barriers, however, the greater implication seems to be that MT1-MMP has fibrinolytic action (224). On the other hand, matrilysin (MMP7) induces endothelial cell proliferation, and up-regulates in cell forming new tubes expressing MMP1 and MMP2 "*in vivo*" (225,226). Other MMPs are involved in cutting the VE-cadherin ectodomain; this provokes cell-cell adhesion inhibition and consequential acquisition of the migratory phenotype from endothelial cells (227). When endothelial cells are induced mechanically to acquire a migratory phenotype, they also express seprase and DPP4 on their surface (7,228). Proteolytic enzymes expressed during angiogenesis exert their functions on several substrates. Basal lamina type-IV collagen is processed by MMPs, generating cryptic alpha v beta 3 binding sites, which induce angiogenesis; this is, also, associated with a loss of binding to integrin alpha 1 beta 1 and an increase in alpha v beta 3 binding and correlates with increased MMP2 expression and activation. The different organization of basal lamina and the exposure of different sites was observed during angiogenesis and in formation of tumor blood vessels, but not in quiescent vessels (229).

The better researched serine proteases involved in angiogenesis are those of the plasminogen activation system, consisting generally of three members: the plasminogen and its two activators- the urokinase-type plasminogen activator (uPA) and the tissue-type plasminogen activator (tPA). Both of them transform the plasminogen into the broad specificity protease plasmin. Compared to other proteolytic cascade systems, the plasminogen is relatively simple in terms of proteolytic enzymes implicated (161); however, the complexity in this system arises instead from the dissimilar mechanisms that control the activity of implicated enzymes. One of the best characterized mechanisms is that offered by the receptor of uPA (uPAR) (230).

TTSP function relates to the proteolytic degradation of extracellular matrix components during tissue remodeling, which play a pivotal role in normal and pathological processes including wound healing, inflammation, angiogenesis, cancer invasion and metastasis. Proteolytic enzymes may activate, or release, growth factors from the ECM or act directly on the ECM itself, thereby facilitating angiogenesis and cell invasion. DPP4 and seprase exhibit both dipeptidyl peptidase activity and a gelatinase activity capable of degrading denature collagens or other ECM components that belong to the new ECM-degrading serine protease family rather than the matrix metalloprotease family (231). Serine proteases and MMPs implicated in new vessel formation are released and/or induced from infiltrating inflammatory cells, as tumor cells and endothelial cells themselves. Different angiogenic factors, bFGF and VEGF, can induce the expression of proteases of both families in endothelial cells; the proteolytic enzymes in stimulated cells localize to the specialized plasma membrane domain, as cell plasma

membrane protrusions (lamellopodia, invadopodia) and shed membrane vesicles (228,232,233). Both MMP2 and MMP9, constitutively secreted by endothelial cells, over-express under induction of bFGF; on the other hand, VEGF induces the over-expression of MMP1. Endothelial cells exposed to type-I collagen, in two and three dimensional culture conditions, up-regulates expression of MT1-MMP, MMP2 and MMP13 and decreases the expression of TIPM-2 (234,235), as well as the over-expresses the serine integral membrane proteases, seprase and DPP4, in endothelial sprout membrane in 3D systems (7). On the other hand, endothelial cells cultured inside to fibrin gel over-express MT1-, MT2-, and MT3-MMPs together MMP2 (236).

Angiogenic growth factors induce differential expression of MMPs and serine proteases by endothelial cells and stroma cells; dissimilar proteolytic enzymes release growth factors form their anchorage to ECM components. Finally the action of proteolytic enzymes on ECM components generate positive and negative regulative peptides as laminin peptides (237,238), the tumstatin a proteolytic fragment of collagen type IV, endostatin and angiostatin, the last two results by proteolytic digestions of collagen type-XVIII and plasminogen respectively (239,107,108), and many orther.

## 8. PERSPECTIVE

During solid tumor formation, angiogenesis is activated to generate a vessel network necessary to respiration, nutrition and growth of tumor mass; to block it appears today to be one of the best therapy strategies. All classes of molecules described (ECM, cadherins, integrins, FAs, and proteases) are involved, in a very complex synergic and/or antagonistic network of signals, in angiogenesis. Their inhibition/inactivation is considered a good strategy to slowdown the pathological process.

ECM could be considered an inducer of angiogenesis, by interstitial collagen, laminin fragments proteolytically generated, etc.; on the other hand, products of ECM are important inhibitors of angiogenesis, they enclose endostatin, a proteolytic fragment due to collagen XVIII digestion, as well as tumstatin derived from collagen type IV and angiostatin from plasminogen digestion. Both ECM activators and inhibitors of angiogenesis are targets of integrins, these molecules in cooperation with focal adhesion molecules transduce positive and/or negative angiogenetic signals. However, angiogenesis is also regulated by endothelial cell-cell adhesion molecules that exert their role not only beyond their pro-adhesive activity; but encompass diverse aspects of vascular cell biology. In particular, the ability of endothelial cadherins to interact with signaling molecules, as catenins, receptor tyrosine kinases, phosphatases, etc. is certainly involved in regulation of cell growth, survival, migration and morphogenesis.

Not in the least, is the role played by proteolytic enzymes and their inhibitors on different substrates. Endogenous protease inhibitors, described above, have been reported to have inhibitory effects on angiogenesis both "*in vitro*" and "*in vivo*". For example, it has been demonstrated that TIMP-3 over-expression induces apoptosis in a variety of cell types and can inhibit vascular neointima formation "*in vivo*"; even if little is known about the mechanisms underlying TIMP-3-mediated apoptosis (240). The TIMP-4 inhibitory effects on endothelial cell tube formation in Matrigel has been reported, while; an opposite effect has been observed in over-expression of TIMP-1 that increases VEGF expression in mammary carcinoma cells and enhance VEGF-induced neovascularization in the retina (241). Concerning serine protease inhibitors, serpins, for example PAI-1 regulator of the plasminogen activator is an essential factor regulating physiological thrombotic/fibrinolytic balance "*in vivo*", but it is also a controller, with integrins, of cell adhesion and motility; and for numerous reasons considered a regulator of tumor invasion and metastasis, such as tumor angiogenesis (242). Also the mapsin, a tumor suppressor gene, silenced in tumor cells, has serine proteases inhibitory activity and anti-angiogenic effects (243).

Because of the roles played by proteolytic enzymes and their inhibitors in angiogenesis regulation, several synthetic inhibitors have been generated. First, production of synthetic MMP inhibitors (MMPIs) were based on the collagen-peptide backbone containing a zinc-binding hydroxamate moiety, which acts on the catalytic site, blocking the enzymatic activity. N-Biphenyl sulfonylphenylalanine hydroxiamic acid (BHPA) but not its enantiomer, inhibits tumor-induced angiogenesis "*in vivo*", inactivating MMP2, MMP9, and MT1-MMP (244). The KB-R7785, batimastat or BB-94, that are peptidomimetic MMPIs, and prinomastat or AG3340, tanomastat or BAY 12-9566, metastat or Col-3, a shark cartilage extract (neovastat or A-941), and several other artificial inhibitors, inhibits the activity of numerous MMPs, plus many of them also disturb the function of VEGFR-2 (245). In addition, serine proteases inhibitors regulate angiogenesis, and apart from the described physiologic inhibitors, several synthetic inhibitors have been synthesized. At this time a number of specific inhibitors have been described and the halomethyl dihydrocouramins have been shown to act as the first general suicide inhibitors of serine proteases (246). The originally described component of the family of couramin inhibitors was halomethyldihydrocouramin, and it was assembled in consideration of the properties of 2-acetoxy-5-nitrobenzylchloride (247). In recent times, the couramin-type inhibitors have evolved as halomethylated dihydrocouramins, having a simpler synthetic pathway and higher efficiency due to an alkyl, aryl ester, thioester, amide o ketone function in position 3 and an electrophilic moiety in position 6 (248-250). The evolution of these compounds are the isocouramins, in which a lactone function could be attacked by nucleophilic serine (251,252).

As for proteases, syntetic molecules with inhibiting integrin function were also generated. For example, the synthetic cyclic peptide mimicking RGD that blocks both alpha v beta 3 and alpha v beta 5 functions, namely Cilengitide (from Merck KgaA, Darmstadt,

Germany) (253). Plus, the cyclic peptideometic compound that also recognises both alpha v beta 3 and alpha v beta 5, SCH221153, and inhibits binding to vitronectin has also been successful in inhibiting angiogenesis in murine tumour models (254,255). However, some laboratories have demontrated that antagonists of alpha v beta 3 integrin have inhibitory effects on the function of other integrins such as alpha 3 beta 1 or alpha 5 beta 1 (256) and can induce angiogenesis (257).

Together with the physiological and/or synthetic inhibitors of angiogenesis, an immunological approach is emerging to block the function of involved molecules and, as a result, new vessel formation and tumor growth. Regarding proteolytic enzymes, monoclonal antibodies to MT1-MMP, which inhibit its enzymatic activity and thus activation of proMMP-2, have been shown to inhibit endothelial cell migration and invasion of collagen and fibrin gels (258). Thus monoclonal antibodies against DPP4, inhibiting enzyme-substrate interaction, regulate endothelial cell migration in collagenous matrix (7,259) The humanized version of the mAb F19 recognizing the TTSP FAP-alpha, called sibrotuzumab, is well-tolerated, not dangerous and it has been demonstrated, by labeling with $^{131}$I, to specifically accumulate in tumor and not in normal tissues. Currently, it is in phase I and limited phase II trials (260,261).

A functional antibody LM609 and its humanized form Vitaxin have been generated to inhibit alpha v beta 3 integrin developed at the Scripps Research Institute, La Jolla, CA, and licensed to Applied Molecular Evolution, San Diego, CA, and Medimmune, Inc., Gaithersberg, MD, USA (Gutheil *et al.* 2000). Also mAbs with inhibitory effects on angiogenesis have been generated against VE-cadherin. Some of them recognizing the extracellular repeat EC-1 were able to prevent formation of stable adhesive structures; but, as just reported, they were active at low doses to inhibit angiogenesis, while at higher doses these mAbs increase vascular permeability and consequently have toxic effects (56). Two other mAbs, BV14, recognizing an epitope in the repeat EC-4 and considered to be of minor importance for VE-cadherin homophilic adhesion, and E4G10, whose epitope was mapped to the first 10 amino acids of the EC-1 repeat, were reported to disrupt endothelial junctions in growing vessels without affecting stabilized junctions in normal vessels (55,262-264). However, initial clinical trials using physiological, synthetic and immunologycal inhibitors of the molecules described as cancer treatments did not show efficacy in terms of reducing tumor progression; in all likelihood this is partially due to the fact that most trials were done in patients with advanced disease stages, when the tumor vasculature is already well-established, and also to the fact that more than one molecule of each family plays multiple roles both in angiogenesis and tumor progression.

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