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Review

Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead

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Sprouting angiogenesis is an invasive process that involves proteolytic activities required for the degradation of the endothelial basement membrane, cell migration with removal of obstructing matrix proteins, and generation of space in the matrix to allow endothelial cells to form a proper lumen. In the last decade it has become clear that besides these matrix-degrading properties, proteases exert additional, more subtle functions that play a key role in angiogenesis. These functions are discussed with specific emphasis on membrane type-1 matrix metalloproteinase (MT1-MMP), other MMPs, and the related ADAMs (a disintegrin and metalloproteinase domain). Proteases modulate the balance between pro- and anti-angiogenic factors by activation and modification of growth factors and chemokines, ectodomain shedding with accompanied receptor activation, shedding of cytokines from membrane-bound precursors, and generation of (matrix) protein fragments that inhibit or activate angiogenesis. Furthermore, they participate in the recruitment of leukocytes and progenitor cells, which contribute to the onset and progression of angiogenesis. Proteases facilitate the mobilization of progenitor cells in the bone marrow as well as the entry of these cells and leukocytes into the angiogenic area. The interaction between pericytes and the newly formed endothelial tubes is accompanied by silencing of MMP activities. Better understanding of the various activities of proteases may be helpful in developing more specific inhibitors that could result in tailor-made modification of proteolytic activities in disease.

1. Introduction

Many physiological and pathological conditions, such as development and growth, inflammation, tissue repair and tumour growth, induce a neovascularization response in order to cope with the increased oxygen and nutrient demand of the tissue.[1] This response starts when the induction of pro-angiogenic factors get the mastery of the anti-angiogenic factors that are present in the tissue, and is recognized as sprouting of microvascular vessels which finally organize in a new perfused vascular bed.[2] In a number of pathological conditions, in particular in tumours, massive angiogenic sprouting occurs, but the process remains stuck in the expansion phase resulting in a disorganized and poorly perfused neovasculature.[3] The process of angiogenesis is accompanied by the recruitment of circulating cells, which contribute to the neovascular growth by providing growth factors and occasionally by insertion of endothelial (progenitor) cells into the new vascular sprouts.[4,5]

Because sprouting angiogenesis is an invasive process, proteolytic activities are required.[6,7] They are needed for the degradation of the basement membrane of endothelial cells allowing invasion into the tissue; for cell migration and removal of obstructing matrix proteins; and for creating space in the matrix to allow generation of endothelial cell tubules. However, in the last decade it has become clear that besides their substrate-specific destructive properties, proteases exert more subtle functions that play a key role in angiogenesis, including the activation and modification of growth factors, cytokines and receptors, and the generation of matrix protein fragments that inhibit angiogenesis.[6–9]

These effects of proteases are most prominently executed by proteases that act outside the cell, in particular at the cell surface, although several proteases within the cell contribute indirectly to angiogenesis by mediating apoptosis, as well as by proenzyme activation and proteasomal degradation, e.g. hypoxia-inducible factor. All major classes of proteases harbour members that are involved in pericellular proteolysis, as schematically summarized in *Figure 1*. It is likely that these proteases often act in larger multiprotein complexes, which define their proteolytic activities in time and space.

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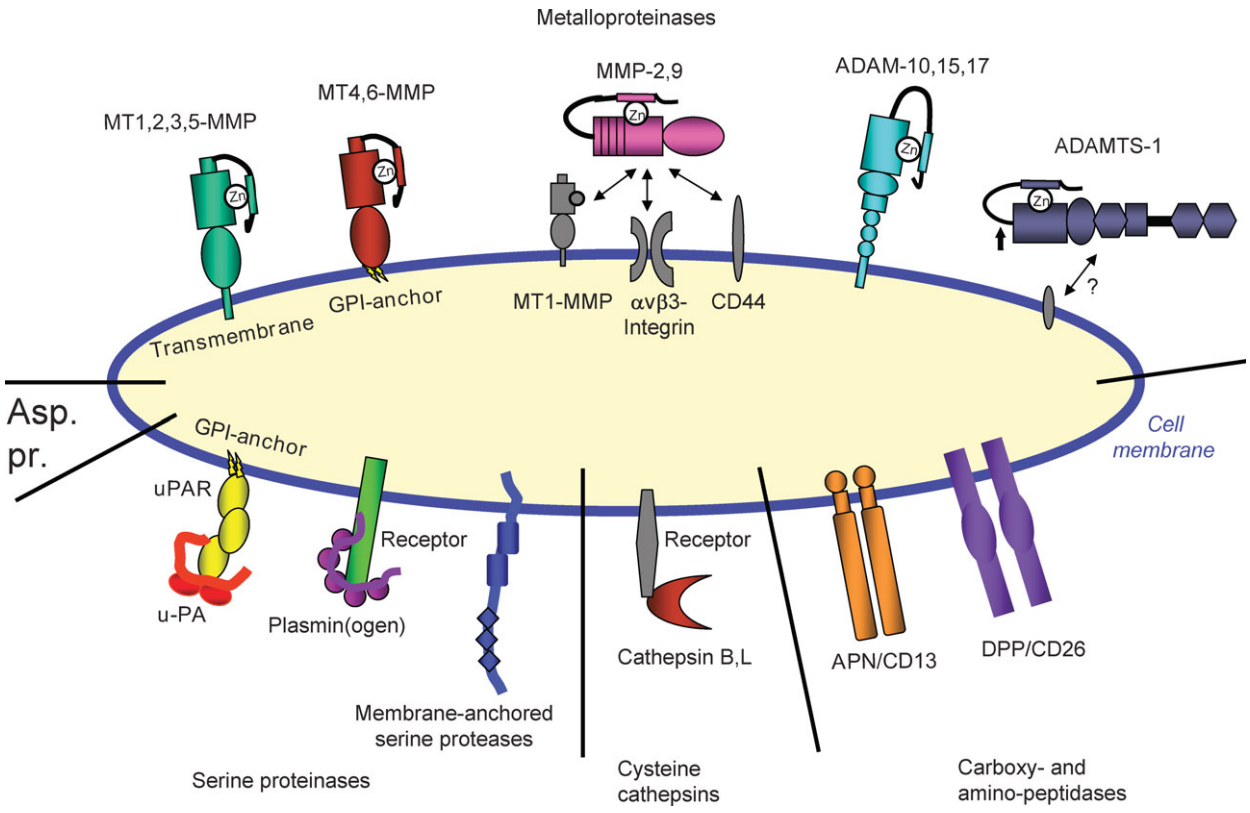

Figure 1 Various classes of proteases contribute to pericellular proteolytic activity that accompanies cell migration and angiogenesis. A selection of members of these classes is depicted. In addition to the four classes of endoproteinases, several members of the amino- and carboxy-peptidase can contribute. Proteases are bound either to the membrane as integral membrane proteins, or to the outer leaflet of the membrane via a GPI-anchor, or to other membrane proteins (receptors). Asp. pr., aspartate proteinases; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; u-PA, urokinase-type plasminogen activator; uPAR, u-PA receptor; APN, aminopeptidase N; DDP, diaminodipeptidase IV; GPI, glycosyl-phosphatidylinositol.

In the present review we focus on new developments with regard to matrix metalloproteinases (MMPs) and related metalloproteinases involved in angiogenesis. For a general overview of the role of other pericellular proteases in angiogenesis the reader is referred to previous reviews.[6,7,10–13]

2. Matrix- and related-metalloproteinases

2.1 Matrix metalloproteinases and related proteinases

MMPs belong to a multigene family of zinc-containing endopeptidases.[14–16] Twenty-three different MMPs have been identified in man (24 in mice), of which endothelial cells can produce many. They comprise secreted MMPs and membrane-type MMPs (MT-MMPs). The latter group consists of four members with a transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) and two with a GPI-anchor (MT4- and MT6-MMP). Together, the MMPs have a broad spectrum of activities and are capable of degrading all known mammalian extracellular matrix proteins (see[6,8,14–16] for details on individual substrate specificities). Several MMPs, in particular MMP-2, MMP-9 and MT1-MMP, have been recognized as crucial regulators for angiogenesis.[6,7,17]

Quiescent endothelial cells produce little or no active MMPs, but these proteases are strongly induced and subsequently activated in capillary sprouts during wound healing, inflammation and tumour growth[6,7,17] and in activated endothelial cells *in vitro*.[18] MMP activities are controlled by a group of endogenous inhibitors known as TIMPs (Tissue Inhibitors of MetalloProteinases) and by RECK (REversion-inducing Cysteine-rich protein with Kazal motifs).[14,17,19] Furthermore, MMP activities on the cell surface are regulated by their activation (removal of their propeptide), complex formation with other proteins, and cellular internalization.[20]

Studies with MMP inhibitors and subsequent cloning of metalloproteinase homologues have revealed two large additional families, of which several members have important pericellular proteolytic activities, the ADAMs (a disintegrin and metalloproteinase domain) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs).

2.2 A disintegrin and metalloproteinase domain

The ADAMs family has 25 members in man, of which 12 have proteolytic activity.[17,21,22] Eleven additional ADAMs are expressed in the mouse. They have a membrane-spanning domain and can act as sheddases.[23] In particular ADAM-10 and ADAM-17 are involved in the release of ectodomains of membrane proteins, which results not only in removal of membrane receptors, but also in the generation of new biological active molecules and in the activation of specific receptors involved in neovascularization, such as Notch and Tie-1 (discussed later).

ADAMs can also act interact with membrane proteins via non-catalytic domains. The disintegrin-like domains of

various ADAMs perform as ligands for various integrins and thus modulate cell adhesion.[24] Similarly, ADAM-15 colocalizes with VE-cadherin, a major structural and regulatory protein of endothelial adherence junctions.[25] ADAM-15 is required for angiogenesis during the development of retinopathy of prematurity in mice, but its deficiency did not affect tumour angiogenesis.[26]

2.3 A disintegrin and metalloproteinase with thrombospondin motifs

A second group of MMP homologues is the ADAMTS family, which has 19 members in man.[22,27] They have proteolytic activity and are produced as secreted proteins, many of which bind to matrix proteins. ADAMTS-1 is dispensable for angiogenesis during murine development.[28] However, in specific conditions ADAMTS-1 and -8 reduced vascular endothelial growth factor (VEGF)-enhanced angiogenesis, such as in the chick chorioallantoic membrane and basic fibroblast growth factor (bFGF)-enhanced angiogenesis in the cornea pocket (see[29] for review). While earlier attention has been given to the potential inhibitory role of two thrombospondin-domains in the C-terminal part of ADAMTS-1,[29] recent data show that ADAMTS1 cleaves thrombospondin-1, and thereby generates anti-angiogenic peptides.[30]

2.4 Endogenous inhibitors of metalloproteinases

The importance of metalloproteinases in angiogenesis is underlined by the impact of deletion of an inhibitor of metalloproteinases RECK. RECK is a GPI-anchored glycoprotein and therefore localized at the membrane surface. It regulates the activities of at least MT1-MMP, MMP-2, MMP-9, and ADAM-10.[31,32] Deficiency of RECK caused disrupted vascular development and premature death of mouse embryos. This pathology is likely caused by uncontrolled MMP activity, as mice those were deficient for both RECK and MMP-2 were partially rescued.[31]

In addition, four mammalian TIMPs exist, which have overlapping activities with respect to their inhibition of most soluble MMPs. More specificity exists regarding MT-MMPs, MMP-19 and proteolytically active ADAMs and ADAMTSs. TIMP-2 and TIMP-3 are good inhibitors of MT1-MMP, MMP-19, and ADAM-17, while TIMP-1 is poor in this respect.[33] The mutual substitution of TIMPs may explain the survival of mice deficient of TIMP-1 or TIMP-3, although TIMP-3-deficient mice developed a dilated cardiomyopathy.[34] Only TIMP-3 is sequestered to the cell membrane and the extracellular matrix, to which it binds with both its amino- and carboxy-terminal domains.[35] At the cell membrane it associates with the glycosaminoglycan, in particular, heparan sulphates, and may play a role in the regulation of ADAM-17.[36]

TIMPs also have biological effects unrelated to metalloproteinase inhibition. TIMP-3, but neither TIMP-1 nor TIMP-2, is involved in binding to the VEGF receptor-2 (KDR) and competes for de binding of VEGF to this receptor.[37] Overexpression of TIMP-3 can induce apoptosis. TIMP-1 and TIMP-2 display anti-apoptotic properties and indirectly induce cell signalling.[38,39]

3. Pivotal role of matrix metalloproteinase and membrane-type matrix metalloproteinase in angiogenesis

A number of studies including gene deletions in mice have pointed to the essential role of MMP-2, MMP-9, and MT1-MMP in the onset of angiogenesis in tumours, development and bone formation.[40–42] Evidence has been provided that matrix degradation is an important aspect in their ability to stimulate angiogenesis. However, it should be noted that the activities of these proteases are complex and may include other pro- or anti-angiogenic effects as well, such as the activation of growth factors and cytokines, degradation of inhibitors, the recruitment of endothelial progenitor cells, and the generation of angiogenesis-inhibiting matrix-derived peptides (detailed later). The impact of this complexity was illustrated with MMP-9. MMP-9 not only stimulates the onset of tumour angiogenesis, but subsequently also generates angiogenesis inhibitors, such as the collagen type-IV-derived fragment 'tumstatin', by which angiogenesis becomes retarded.[43] Whether MMP-19, which acts as a negative regulator of early steps in tumour angiogenesis and invasion,[44] acts in a similar way is not yet known.

When the abilities of various proteases to enhance capillary-like tube formation in a collagen-rich matrix were compared on the basis of deletion experiments, only MT1-MMP appeared indispensable for endothelial cells to form invading tubular structures, while MMP-2, MMP-9, their cognate cell-surface receptors β3-integrin and CD44, or plasminogen were not.[45] However, single gene deletions only identify those proteases that have unique properties, whereas proteases with crucial properties that are shared with comparable proteases may not be identified.

The complex role of MMPs and related proteases in the regulation of angiogenesis are further discussed according to their involvement in various aspects of the angiogenesis process.

4. Proteases and the pro/anti-angiogenic balance

Angiogenic growth factors and inflammatory cytokines can induce a number of pericellular acting proteases, including MT1-MMP, MMP-2, MMP-9, and u-PA. This is generally seen as a part of the repertoire of cellular activities that is switched on when proangiogenic growth factors have overruled angiogenesis-inhibiting factors. However, this unidirectional view on the relation between angiogenic growth factors and proteases has evolved into the insight that proteases themselves also contribute to fine-tuning of the activities of various growth factors that control the onset and progression of angiogenesis.[6,7,9]

4.1 Activation and modification of growth factors

Several growth factors involved in angiogenesis require proteolytic processing to become active. Hepatocyte growth factor (HGF) is activated by the serine protease HGF activating factor and the transmembrane serine proteases matripsin and hepsin.[46] TGF-β can be activated by the action of plasminogen activators and thereby becomes possible to modulate angiogenesis.[47,48] Heparin-binding epidermal

growth factor is released from its membrane bound precursor by ADAM-17.[49]

Growth factors can be kept in complex with other proteins or proteoglycans, and become liberated by degradation of their binding moieties. VEGF is inactive in complex with connective tissue growth factor (CTGF), but becomes active again after proteolytic cleavage of CTGF by MT1-MMP, or MMP-1, -3 or -13.[50] In addition, growth factors with heparin-binding properties (bFGF, VEGF) can be liberated from matrix proteoglycans by heparinases and proteases.[51]

Binding of growth factors, such as VEGF, to heparan sulphates does not only create a pool of growth factors, but also a specific pattern of VEGF availability, which guides invading endothelial cells to form proper new microvessels. When this guidance is lost, chaotic vessels may be formed. This occurs when MMP-3 or MMP-9 cleave $VEGF_{165}$ and generate a shorter non-heparin-binding active form of VEGF, that, comparable to $VEGF_{121}$, induces an irregular vessel pattern.[52] Similarly, the properties of stromal cell-derived factor-1 (SDF-1) are modified by carboxy- and amino-terminal truncations (discussed later).

4.2 Ectodomain shedding and receptor signalling

The shedding of ectodomains of receptors and other membrane proteins has been recognized for a long time, but only during the last years it has become clear that ectodomain shedding is an initial step of the activation of specific receptors, such as Notch, ErbB-4 and Tie-1.[53] Notch-1 signalling plays a role in endothelial differentiation and embryonic, and tumour angiogenesis,[54] while the angiopoietin receptor Tie-1 is indispensable for embryonic blood vessel formation.[55]

Binding of the ligands DeltaL4 and Angiopoietin-1 to Notch-1 and Tie-1, respectively, facilitates the proteolytic cleavage of the ectodomains of these receptors by ADAM-17 and ADAM-10.[56,57] After this initial proteolytic step, the remaining transmembrane and intracellular part is further processed intracellularly by the γ-secretase complex, in which the aspartate proteinase presenilin is the active protease.[58] By this cleavage the cytoplasmic fragment of the receptor becomes available for cellular signalling and, in the case of Notch, transfers to the nucleus. Deficiency of ADAM-10, but not that of ADAM-17 resembles the early embryonal pathology of Notch1/Notch4 double knock-out mice suggesting a dominant role for ADAM-10 in mice.[57]

4.3 Shedding of other proteins

Various cytokines and growth factors are produced as an extracellular part of a membrane integrated proform. After proteolytic shedding, these factors perform their paracrine action. TNFα is released by ADAM-17, also known as tumor necrosis factor (TNF)-converting enzyme. Furthermore, by shedding of EphA2 and EphA3 ADAM-10 generates cleaved soluble Eph receptors, which inhibit tumour angiogenesis in mice.[59] Other examples are the shedding of pro-angiogenic soluble E-selectin,[60] shedding of urokinase receptor (uPAR) by MMP-12,[61,62] which generates a cleaved soluble-uPAR with chemotactic properties for leukocytes and progenitor cells (discussed later), and shedding of IL-6 receptors by ADAM-10 and ADAM-17,[63] which facilitates IL-6 signalling in endothelial cells.[64] MT1-MMP can shed soluble Semaphorin-4D from its membrane-bound form on tumour cells, which interacts with plexin-B1 on endothelial cells and induces pro-angiogenic chemotaxis of these cells.[65]

These examples suggest that the shedding of ectodomains from membrane proteins is a rather general mechanism. As potent biological modifiers can be generated, it is likely that proteolytic shedding is a tightly controlled mechanism. In addition to several ADAMs and MT-MMPs, membrane-bound serine proteases[13] may be involved.

4.4 Endogenous inhibitors generated by proteases

Invasive growth and angiogenesis are accompanied by proteolytic degradation of matrix proteins. Among the proteolytic degradation products derived from extracellular matrix proteins and haemostasis factors a number of fragments have potent angiogenesis inhibiting properties.[6,9,66,67] A fragment of thrombospondin was initially recognized as a potent angiogenesis inhibitor. Subsequently, Folkman and co-workers identified fragments of plasminogen and collagen-XVIII, angiostatin, and endostatin, as tumour-derived factors that suppressed the growth of metastases by potent angiogenesis-inhibiting properties. Later studies revealed a range of angiogenesis-inhibiting (and some stimulating) fragments of matrix proteins and coagulation factors, such as tumstatin (collagen-IV), alphastatin (fibrinogen), kininostatin (HMW-kininogen) and many others. These fragments are also indicated as matrikines and have been the subject of various reviews.[6,9,66]

5. Pericellular matrix metalloproteinases and invading capillary sprouts

Once properly activated, endothelial cells degrade their basement membrane and start migrating, guided by factors produced by leukocytes or tissue cells. The invasion into a tissue matrix requires surface-bound proteolytic activities to enable the cell to migrate along matrix proteins, to remove matrix fibres that obstruct invasion of the cell body, and to widen the initial sprout into a true microvessel. An exceptional case of endothelial migration represents the re-growth of endothelial cells into casts of basement membrane, which remained present after anti-angiogenic treatment.[68] After cessation of the treatment endothelial cells migrate into such preformed basement membrane casts much faster than into a normal matrix. Probably, the presence of growth factors in these casts and the absence of obstructing matrix proteins facilitated migration.

The temporal and spatial expression of specific proteases and their activities must be well controlled to avoid endothelial cell detachment and apoptosis. Proteolysis is limited to the cell surface and fine-tuned by interaction with receptors and inhibitors. Various cell-bound proteases contribute to endothelial cells invasion, including MT-MMPs, aminopeptidase CD13, and receptor-bound u-PA, plasmin, MMP-2, MMP-9, and cathepsin-B and -L[7,69–73] (*Figure 1*). Notwithstanding the potential contribution of other proteases, MT1-MMP is generally considered as the most prominent player in pericellular proteolytic activity[20,74] and is essential in migration of cells in type-I collagen.[45,75] The next section focuses on the regulation of MT1-MMP activity in cell migration and invasion involved in angiogenesis. Examples

of other proteases, such as the cell-bound u-PA/plasmin and cathepsins are reviewed elsewhere.[7,70,76]

5.1 Membrane type-1 matrix metalloproteinase

First recognized MT1-MMP in 1994, MT1-MMP has rapidly developed to the central position in the regulation of pericellular proteolytic activity.[20] Its molecular structure consists of extracellular catalytic and haemopexin domains, a transmembrane domain and a short cytoplasmatic tail. The catalytic domain is activated during transport from the Golgi compartment to the plasma membrane by removal of the propeptide by furin or a furin-like serine protease. Together with the haemopexin domain it is involved in the proteolytic activity of MT1-MMP and its TIMP-2-dependent activation of MMP-2.[15,20]

MT1-MMP is induced in endothelial cells by HGF,[77] thrombin,[78] VEGF and TNFα[79] and by *Egr-1* mediated transcription induced by interaction of the cell with a 3D collagen matrix.[80] Shear forces and angiopietin-1/Tie-2 signalling suppress its transcription.[75,81]

5.2 Membrane type-1 matrix metalloproteinase at the invading front

There is ample evidence that MT1-MMP is present at the leading tip of invading cells.[20,74,82] However, how can proteolytic activity at the cellular front help a cell to move forward? The cell makes cellular protrusions with integrin-mediated matrix contacts to enable it to pull itself forward. However, once this process is initiated, cell movement will be hampered by existing cell-matrix interactions, which keep the cell fixed in its original position, or by the extracellular matrix meshwork, usually collagen, that allows entrance of the thin cellular protrusions, but not the thicker cell body.

For tumour cells invading a 3D-collagen matrix, Wolf *et al.*[83] observed that, while MT1-MMP is present at both the anterior adhesive and posterior proteolytic zones of the leading edge of invading cells, its collagenolytic activity was only present at the latter zone (compare *Figure 2*). This enabled the cell to generate pulling force between the leading end and the cell body without loosing grip, while movement was allowed in the adjacent area by proteolytic activity. Proteolytic activity was particularly present at sites where the existing collagen fibres interfered with the forward movement of the cell.[83]

Such a mechanism requires that MT1-MMP activity will be masked until it has moved from the tip of the protrusion to the adjacent proteolytic zone. Mechanisms that may contribute to such masking are a two-step proteolytic processing,[84] which delays activation after initial cleavage by furin; temporary binding of inhibitors, like TIMP-2 and RECK; or delay of MT1-MMP dimerization,[85] which is needed for MMP-2 activation and cell migration. Preferential binding of pro-MMP-2 to obstructing collagen threads may add to localizing the combined MT1-MMP/MMP-2 proteolytic activity to sites where collagen is in close contact to the cell and hampers cell movement.

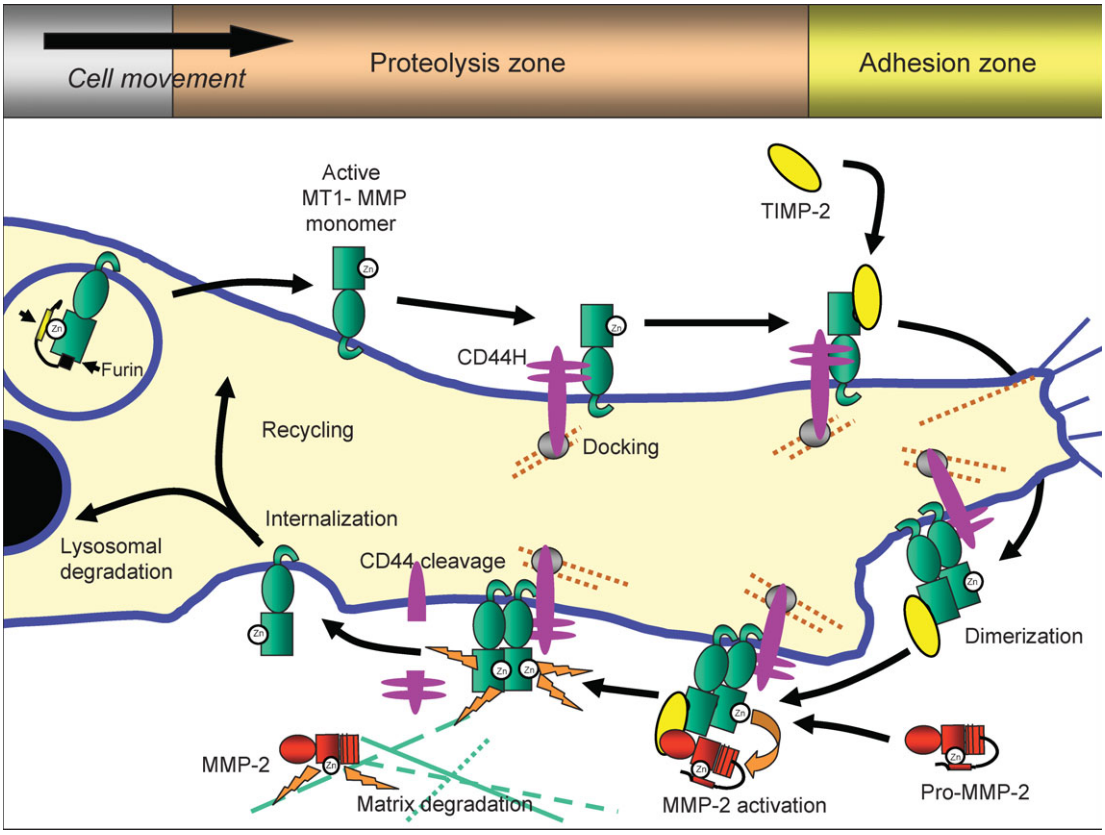

Figure 2 Schematic presentation of the activation, activity, and disappearance of membrane type-1 matrix metalloproteinase (MT1-MMP) at the surface of the invading endothelial sprout. MT1-MMP is activated and exposed at the cell surface in the vicinity of the invading sprout. It should be noted that the steps that are depicted at the upper part of the invading front of the cell, actually are concentrated at the leading front, as the endothelial sprout has no luminal-abluminal polarity. The division in adhesive and proteolytic zone is based on data in tumour cells (see text for details).[103]

Once MT1-MMP activity is available, both MT1-MMP and MMP-2 can degrade various matrix proteins.[6,20,74] MT1-MMP has a broad-spectrum proteolytic activity towards a variety of extracellular matrix proteins including collagens, fibronectin and laminin-5. Its ability to activate MMP-2 and MMP-13 extends its effect on matrix degradation.[15,20] Furthermore, MT1-MMP activity can cause enhanced intracellular activation of sarcoma (Src) and extracellular-related kinase (ERK) and VEGF gene expression.[86,87]

5.3 Internalization of membrane type-1 matrix metalloproteinase

One of the additional substrates for proteolysis by MT1-MMP is CD44H, which binds both to the haemopexin domain of MT1-MMP and indirectly to the actin cytoskeleton.[74,88,89] The potent proteolytic activity of MT1-MMP at the cell surface is self-limiting, as MT1-MMP is subsequently internalized. This process is inhibited when the cleavage of CD44H is prevented by mutation.[88] Once MT1-MMP is internalized it can be degraded in the lysosomes or shuttled back to the plasma membrane depending on further regulatory steps (*Figure 2*). MT1-MMP can be internalized via clathrin-coated vesicles or via caveolae. Galvez *et al*.[90] reported that internalization of MT1-MMP via caveolae was required for the migration of endothelial cells in a collagen substrate. However, in other cells requirement of clathrin-mediated uptake was indicated as main mechanism of uptake, in which the short cytoplasmic tail of MT1-MMP played a role.[91] Whether the different internalization pathway in endothelial cells mainly reflects the abundant presence of caveolae in these cells is not yet known.

Recent data indicated that a Src-dependent phosphorylation at Tyr573 in the short cytoplasmic domain of MT1-MMP was required for sphingosine-1-phosphate-induced cell migration in endothelial cells.[87] Palmitoylation at the adjacent Cys 574 was found to be essential for MT1-MMP-dependent cell migration and clathrin-mediated internalization,[92] suggesting that the cytoplasmic domain must be aligned along the membrane for this activity.

Interestingly, no information is presently available whether MT1-MMP activity disappears during MT1-MMP internalization. A report suggesting an intracellular action of MT1-MMP suggests that this is not necessarily the case.[93]

5.4 Other membrane-type-matrix metalloproteinases

Other MT-MMPs also contribute to cell migration and collagenolysis.[94] Comparable to MT1-MMP, the membrane spanning MT2- and MT3-MMP induced endothelial tubules, while the GPI-anchored MT4-MMP was unable to do so.[94] MT3-MMP, rather than MT1-MMP was involved in the formation of tubular structures by human endometrial microvascular endothelial cells, while their foreskin counterparts used MT1-MMP.[79] MT2-MMP may also be involved in the endometrium.[95] Interestingly MT2-MMP can activate MMP-2 independent of TIMP-2.[96]

6. Proteases and the recruitment of bone marrow-derived cells

There is ample evidence that leukocytes and endothelial progenitor cells can additionally contribute to angiogenesis.[5,97] Monocytes and mast cell progenitor cells produce various pro-angiogenic factors.[97,98] This is also the case with a special population of CD34+ cells that can acquire endothelial-like properties, such as the expression of VE-cadherin and VEGF receptor-2, and tentatively are indicated as endothelial progenitor cells (EPCs). EPCs are thought to accelerate the progression of angiogenesis,[5,99] and their absence or dysfunction has been associated with impaired vascularization in cardiac and diabetes patients.[100] According to recent data, most of these cells are monocytic in nature, and only a small proportion represents real endothelial progenitor cells.[101,102] Several proteases, including MT1-MMP, MMP-9, uPAR-bound u-PA and cathepsin-L, play a role in the mobilization of various types of progenitor cells in the bone marrow and in the recruitment of these cells into the areas of neovascularization.[70,76,103]

6.1 Matrix metalloproteinase-9 and mobilization of progenitor cells

With respect to the mobilization of various types of progenitor cells MMP-9 received special attention. Recruitment of progenitor cells from the osteoblastic niche into the vascular zone of the bone marrow requires Kit-ligand bioavailability.[104] This availability is facilitated by shedding of soluble Kit-ligand (sKitL) from its membrane-bound precursor by MMP-9 mediated proteolytic cleavage. In MMP-9-deficient mice the release of sKitL was impaired, and recruitment of haemopoietic progenitor cells delayed.[104] Similar experiments performed in eNOS-deficient mice demonstrated that nitric oxide (NO) is involved in VEGF-induced MMP-9 activation.[105] Indeed, NO enhances MMP-9 activity.[106] The release of sKitL by VEGF-induced MMP-9 mobilizes endothelial and mast progenitor cells in a comparable way.[99]

6.2 Homing of progenitor cells and entry into the angiogenesis area

Under basal conditions a small proportion of progenitor cells is already released to the circulation. They will be attracted to areas of tissue repair and neovascularization or home back to the bone marrow. Chemokines, in particular SDF-1, play an important role in directing progenitor cells. Proteases enable the progenitor cells to enter a tissue.[76,107–109] Various reports describe the expression of MMP-2 and MMP-9 in peripheral blood and bone marrow CD34+ progenitors as well as cultured EPCs. Stimulation with TNFα, IL-8 or SDF-1 resulted in increased MMP levels, facilitating the migration of EPCs into Matrigel plugs or transwell systems.[107–109] In addition to MMPs, EPC-derived cells produce u-PA/uPAR and various cysteine cathepsins.[76,110]

MMP-12 and several other proteases can release uPAR antigen from the cell surface and cleave uPAR at the linker region between domains 1 and 2 of uPAR.[61,111,112] *In vitro*, the cleaved soluble urokinase-type plasminogen activator (suPAR) was able to chemoattract leukocytes and haemopoietic stem cells by activating the high-affinity fMLP receptor.[113,114] Furthermore, the suPAR-derived peptide uPAR(84–95) inhibited migration of haemopoietic progenitor cells towards SDF-1.[62] *In vivo*, this uPAR peptide increased the number of haemopoietic progenitor cells in peripheral blood, and inactivated murine CXCR4, through an unknown mechanism.[114] Since the SDF-1 receptor CXCR4 facilitates retention of stem/progenitor cells in bone marrow and

peripheral tissue, inactivation of CXCR4 by cleaved-suPAR might explain this mobilizing effect. It is likely that this effect occurs in various types of progenitor cells, as SDF-1 is an important homing factor for all of them.

It is of interest to note that the activity of SDF-1 by itself can also be modified by proteolytic cleavage. The two isoforms of SDF-1, SDF-1a, and SDF-1b, are both modified by the aminodipeptidase DPPIV/CD26, by which their heparan sulphate affinities and interactions with their receptor CXCR4 are reduced.[115] SDF-1a can also be shortened by carboxypeptidase N (also called thrombin activatable fibrinolysis inhibitor),[116] which further reduces the affinity for heparan sulphates. MT1-MMP, MMP-1, -3, -9 and -13 can cleave and thereby inactivate SDF-1 or markedly weaken the binding to its receptor CXCR4.[117]

7. Maturation of newly formed vessels

The present paradigm of angiogenesis considers the smooth-muscle-like pericyte as key regulator of the stabilization of newly formed vessels.[118,119] MMP-9 deficient animals had an impaired pericyte mobilization, which markedly affected the extent and stability of neovascularization of neuroblastomas.[120] The balance between proteolytic activity and proteinase inhibition is important. Pericyte-derived TIMP-3 inhibits MT1-MMP-dependent MMP-2 activation on the endothelial cells, and thus contributes to the stabilization of newly formed microvessels. Endothelial-cell-pericyte co-cultures strongly induce TIMP-3 expression by pericytes, whereas endothelial cells produce TIMP-2.[121] Together, they contribute to vascular stabilization by inhibiting a variety of MMPs, ADAMs, and VEGFR-2. This stabilization involves assembly of basement membrane matrix, which can accumulate TIMP-3 by proteoglycans, and cessation of endothelial sprouting.[121]

Once the new microvessels become perfused, the increased demand on blood supply will induce outward remodelling of the proximal vessels, which requires MMP activities as well.[20]

8. Perspective

This review has mainly been focused on MMPs and ADAMs, of which several members play a pivotal role in angiogenesis. MMP-2, MMP-9 and MT1-MMP and their inhibitor RECK are a prerequisite for the development of proper blood vessels. Their deficiencies result in death or major defects during embryonic or perinatal development. Similarly, deficiencies of other proteases including the serine proteases prothrombin, factor VII and matriptase-3 result in impaired embryonic development. ADAM-10 and ADAM-17 also play crucial roles particularly in membrane protein shedding and the activation of receptors such as Notch and Tie-1.

Proteases can also be involved in (patho)physiological processes without being essential for unchallenged development. Many proteases play a crucial role in the regulation of a proper response to various stresses, such as acute hypoxia, inflammation and wounding. Their involvement becomes visible if the specific stress is exerted, but without this stress their presence can be dispensable. For example, u-PA deficiency, as well as inhibition of MMPs, result in a retarded vascularization in mouse ischaemic heart.[122] Deficiency of MMP-13 retards bone fracture healing and its associated angiogenesis.[123] Aminopeptidase-N deficiency affects neovascularization in oxygen-induced retinopathy.[73]

Proteases and their products contribute to the fascinating complexity by which the formation and adaptation of the vascular system is regulated. Proteolytic activities during matrix remodelling and cell migration have been recognized earlier and indicated as targets for therapeutic intervention in rheumatoid arthritis and tumours.[124] However, initial attempts were unsuccessful, because understanding the contributions of proteases was incomplete.[125] Modification and generation of growth factors and cytokines, ectodomain shedding and receptor signalling, and generation of matrikines are new areas, which only recently received the attention that they deserve. Better understanding of the various activities of proteases and development of more specific inhibitors may help designing more tailor-made treatments.

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