Progenitor Cells and Hypoxia in Angiogenesis

Verloop, R.E.

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Proteases and Angiogenesis

R.E. Verloop
V.W.M. van Hinsbergh

Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, the Netherlands

ABSTRACT

The classical view on extracellular proteases as being merely degrading proteases paving the way for the new vessel and its lumen has markedly extended during the last decade. Activation, and tailoring of growth factors, as well as the generation of new biological mediators derived from matrix and circulating proteins result in new biological activities controlling angiogenesis. This chapter surveys the different families of extracellular proteases contributing to the angiogenic process. Subsequently, it describes how these proteases are involved in various aspects of the neovascularization process. Besides from their 'classical' role in matrix degradation, cell invasion and lumen formation, proteases are involved in the modification of growth factors and receptors, generation of matrix fragments with anti-angogenic properties (matrikines), and the recruitment of bone marrow derived progenitor cells, enforcing angiogenesis. Intracellularly, proteases can also be involved, such as in the activation of hypoxia-inducible factor-1a , Notch-1, and membrane-type-1 matrix metalloproteinase. An overview is given how cells fine-tune proteolytic activity to their proximity during the overall process of new blood vessel formation.

INTRODUCTION

Angiogenesis is the complex process of capillary formation from the pre-existing vasculature, which occurs both in development, wound repair and many pathological conditions, such as cancer, diabetic retinopathy and arthritis[1]. In addition to growth factors and matrix binding cell receptors, proteases are required for the progress and proper control of angiogenesis[2, 3]. Proteases contribute to matrix degradation required for endothelial cell detachment from the basement membrane, cell invasion into the interstitial matrix, and lumen formation of the new vascular structures. Furthermore, on the surface of cells they guide cell migration and contribute to the activation and shedding of growth factors and receptors. Finally they modify growth factors and matrix proteins, by which peptides are generated with new biological properties. These actions contribute to the formation of new capillary sprouts, a process that usually is enforced by the recruitment of progenitor cells and leukocytes, which provide *i.e.* additional angiogenic growth factors. When angiogenesis is effective, the new microvascular structures still require an adequate perfusion. To that end, the proximal vascular tree has to be adapted as well. This requires the enlargement of the diameter of arterioles and small arteries by remodeling of their smooth muscle cell layer. Many of the proteases that are involved in angiogenesis-related matrix remodeling and cell migration are also active in this latter process.

These most obvious actions of proteases in angiogenesis regard proteolytic activities in the extracellular matrix and on the surface of the invading endothelial cells. These extracellular actions are the subject of the present chapter. It should be noted that in addition to these extracellular events, some proteases contribute to angiogenesis control by intracellular action, either by processing of proteins or as key regulators in apoptosis and proteasome activities. Processing of proteins involves here the maturation of proteins such as activation of matrix metalloproteinases by furins [4], and intracellular degradation of matrix proteins[5]. Other proteases, the caspases, control apoptosis, an essential process in endothelial death and vascular pruning[6]. Furthermore, an important regulator of angiogenesis is the transcription factor hypoxia-inducible factor-1, of which the a-subunit (HIF-1a) is oxygen-dependently controlled by proteasomal degradation[7]. The expression of many genes that contribute to angiogenesis can be induced by HIF-1, including VEGF, VEGFR-2 (*Flk-1/KDR*), VEGFR-1 (*Flt-1*), stromal cell derived factor-1 (SDF-1), platelet-derived growth factor-ß (PDGF-ß), erythropoietin and basic fibroblast growth factor (FGF-2).

In this chapter we shall focus on the different types of extracellular proteases and their contributions to various aspects of angiogenesis. While the initial interest in the involvement of proteases in angiogenesis was related to their function in matrix degradation, it has become obvious that the functions are manifold. After survey-

ing the various types of extracellular proteases involved in angiogenesis, we shall discuss how these proteases fine-tune proteolytic activities on the leading edge of migrating and invading cells; their role in the recruitment of leukocytes and bone marrow-derived progenitor cells; their contribution to the activation and modification of angiogenic growth factors, which results in altered properties; and their involvement in generating matrix fragments with angiogenesis inhibiting properties, called matrikines. Finally we shall discuss the potential of some proteases to be good candidates as biomarkers for a specific disease, and the perspective and complexity of protease inhibition as a target for therapy in diseases with neovascularization.

EXTRACELLULAR PROTEOLYTIC ACTIVITY IN ANGIOGENESIS

All major classes of extracellular proteases have been mentioned in the regulation of angiogenesis, although their contributions may vary considerably. In particular the serine proteases of the plasminogen activator (PA)/plasmin system and the matrix metalloproteinases (MMP) received much attention as major regulators of cell migration and invasion. More specific functions, e.g. in progenitor cell recruitment, have been attributed to cysteine cathepsins. In addition to these endopeptidases, amino- and carboxy- exopeptidases contribute to the fine- tuning of neovascularization by trimming proteins and altering their biological activities. The next sections briefly survey these groups of proteases.

SERINE PROTEASES

Serine proteases constitute the largest family of proteases that have been identified in the human degradome (the complete repertoire of proteases expressed by a tissue or organism)[8]. They are a heterogeneous group of endopeptidases, which are characterized by the so-called “catalytic triad”, a catalytic side made up by serine, aspartic acid and histidine. Serine proteases are produced as inactive zymogens, requiring proteolytic removal of an N-terminal propetide, shielding the catalytic site. Involvement of serine proteases has been demonstrated in many fields of vascular biology, including the well-known coagulation cascade and fibrinolytic system, the complement cascade, and angiogenesis. Inflammatory cells also yield a significant amount of serine proteases.

A well-studied example of this protease family in vascular remodeling is the plasminogen activator (PA) - plasmin system, consisting of tissue-type PA (tPA), urokinase-type PA (uPA) and receptor (uPAR), and their substrate plasminogen. These proteases are controlled by specific inhibitors and by cellular receptors, such as the uPA receptor (uPAR). Plasminogen, a 92-kD protein, is present in blood as the inactive precursor of plasmin, a pluripotent serine protease. Plasminogen is converted to

plasmin by either tPA or uPA. Plasmin possesses proteolytic activity on a wide range of extracellular matrix components, such as fibrin, collagens, laminin, fibronectin and proteoglycans[9]. In addition, plasmin is capable of activating a variety of latent MMP. Activation of plasminogen by tPA is the major pathway that leads to lysis of fibrin clots in the blood and body cavity. Activation of plasminogen by uPA, facilitated by the uPAR[10], is responsible for mediating plasminogen activation at the cell surface and appears to initiate most of the nonfibrinolytic activities of plasmin, such as cell migration and invasion[11].

Inhibition of the PA-plasmin system occurs either at the levels of PAs, regulated by specific inhibitors (PA inhibitor-1 (PAI-1)) or at the levels of plasmin (a2-antiplasmin (a2-AP) and a2-macroglobulin). Interestingly, a2-macroglobulin, which is the most prominent circulating protease inhibitor, can also inhibit MMP activity, but because of its large size, its effectiveness as an inhibitor in areas of wound healing may be limited[12]. Recent findings reported that plasmin cleaves vascular endothelial growth factor (VEGF) in extracellular matrix. Matsuno *et al*.[13] found that lack of a2-AP enhances the secretion of VEGF in acute myocardial infarction and oversecretrion of VEGF promotes heart failure by pulmonary edema. Moreover, regulation of VEGF by a2-AP significantly affected reendothelialization after vascular injury[14].

Human tissue kallikreins (hK) represent another family of serine proteases, comprising 15 homologous single-chain, secreted serine endopeptidases. Kallikrein 2, 3, 6, 7, and 14 directly catalyse the hydrolysis of a distinct and overlapping set of extracellular matrix proteins, enabling endothelial cell migration and invasion[15-20]. *In vitro* studies demonstrated that hK support angiogenesis by direct or indirect disruption of extracellular matrix barriers. Indirect mechanisms of hK on angiogenesis encompass the activation of pro-MMP2 and pro-MMP9 by hK1[21-23], and activation of the uPA – uPAR system by hK2 and hK4[24, 25]. In addition to stimulating angiogenesis, hK can also antagonize this process. hK3, hK6 and hK13 generate angiostatin-like fragments from plasminogen *in vitro*[26-28]. For a more detailed overview of the role of tissue kallikreins in invasive cancer and angiogenesis, the reader is referred to Borgono *et al*.[29]

MATRIX METALLOPROTEINASES

To date 24 different matrix metalloproteinases (MMP) have been identified, of which 23 are found in man. MMP belong to a multigene family of zinc-dependent endopeptidases, which together are capable of degrading all known extracellular matrix proteins. They enhance cell migration and invasion and contribute to development and growth and adult vascular remodeling, such as occurs in wound healing, arterial adaptation and angiogenesis[30-32]. Their proteolytic activity is not only involved in ma-

trix breakdown, but can also aid in the activation of other MMP and growth factors[2, 33, 34]. The activity of MMP is regulated both at the level of transcription and activation, as well as by a group of endogenous inhibitors known as tissue inhibitor of metalloproteinases (TIMP).

Based on their extracellular positioning they can be arranged in two different groups: soluble MMP and plasma membrane anchored MT-MMP (membrane-type MMP). A further subclassification of the soluble MMP can be made on the basis of sequence similarity, domain organization and substrate specificity, yielding collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26) and a group of less described MMP.

The membrane-type MMP encompass six members, which are bound to the membrane by either a type-I transmembrane domain (MT1/2/3/5-MMP) or a glycosylphospatidylinositol (GPI) anchor (MT4/6-MMP). While most of the soluble MMP are secreted as inactive pro-enzymes and become activated once they are present in the extracellular compartment, MT-MMP are processed intracellular by furin-like enzymes. Initially, the MT-MMP were mainly considered as activators of soluble MMP. However, recent data demonstrated that they are able to degrade matrix proteins by themselves, including fibrillar collagen, laminin-1 and -5, aggrecan, fibronectin, vitronectin and fibrin[33]. This is important in the concept that controlled extracellular matrix degradation has to be limited to the close vicinity of cells. Loss of activity control may result in tissue damage as often seen in diseases like arthritis, aneurysms and cancer[35]. In the well coordinated proteolytic activity on the cell membrane MT-MMP serve their most important role, as is explained below for MT1-MMP in the section on pericellular proteolysis in cell migration.

RELATED METALLOPROTEINASES: ADAMS AND ADAMTS

ADAM (a disintegrin and metalloproteinase domain) and ADAM-TS (with thrombospondin motifs) represent two protein families that are structurally related to the MMP. They can alter the balance between pro-and anti-angiogenic factors by different mechanisms.

The ADAM proteases are membrane bound and act as sheddases releasing growth factors and membrane receptors (see below). ADAM-17 (=TNFa converting enzyme/TACE) proteolytically releases TNFa and HB-EGF from their membrane-bound precursors, and contributes to Notch signaling by liberation of membrane–bound Notch or its ligand Delta[36, 37]. This may affect angiogenesis as Notch signaling is involved in endothelial differentiation and in embryonic and tumor angiogenesis[38, 39]. ADAM-15 has been shown to be required for angiogenesis during the development

of retinopathy of prematurity, but its deficiency did not affect tumor angiogenesis[40].
ADAMTS-1 and -8 reduced VEGF-enhanced angiogenesis in the chick chorioallantoic membrane and endothelial proliferation *in vitro*. They also inhibited bFGF-enhanced angiogenesis in the cornea pocket[41]. Furthermore, ADAMTS-1 and its C-terminal half region suppressed experimental lung metastasis, which was accompanied by reduced tumor angiogenesis in these metastatic lesions[42]. Particularly the thrombospondin (TSP) domains in ADAMTS contribute to the angiogenesis inhibiting properties of ADAMTS proteins (see Iruela-Arispe *et al*. for review[43]).

ENDOGENOUS INHIBITORS OF MMP ADAM

The activity of MMP is regulated predominantly by a group of structurally related, endogenous inhibitors, known as tissue inhibitors of metalloproteases (TIMP), which reversibly bind MMP with a 1:1 stoichiometric ratio[44]. In addition MMP can also be inhibited by a2-macroglobulin and the membrane-anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with Kazal motifs)[45, 46].
Four members of the TIMP family have been identified: TIMP-1, -2, -3, and -4. Their structures are largely conserved, but the C-terminal domains are more variable, contributing to their specificity towards preferred MMP targets[44, 47-49]. Most soluble MMP can be inhibited by all TIMP, but MT-MMP and ADAM have a somewhat more restricted pattern of TIMP inhibition. In contrast to its family members, TIMP-1 fails to inhibit MT1-MMP[50]. TIMP-3 differs from the other TIMP, inasmuch as it is better suited for inhibition for ADAM-17, -10, -12, and the ADAMTS-4 and -5[51-54]. Another unique feature of TIMP-3 is that it can associate with the glycosaminoglycan chains of proteoglycans on the cell membrane[55], where it may play a role in ADAM-17 regulation[56].
Besides from their role in MMP inactivation, TIMP also exert other biological functions. TIMP-2 can suppress basic fibroblast growth factor-induced endothelial cell proliferation *in vitro* and angiogenesis *in vivo*, independent of MMP inhibition[46, 57]. TIMP-3 has a proapoptotic activity, possibly through stabilization of the TNF alpha cell receptor 1, Fas, or by the reported blocking of VEGF to VEGFR-2 binding[58]. On the other hand, TIMP-1 and TIMP-2 have antiapoptotic activity[59-62].

CYSTEINE CATHEPSINS

Human cysteine cathepsins belong to the papain subfamily of cysteine proteases[63]. In man this family of cathepsin cysteine proteases comprises eleven members, namely cathepsin B, C, F, H, L, K, O, S, V, W, X/Z. Cathepsins are predominantly endopeptidases which are located intracellular in endolysosomal vesicles required for terminal protein degradation and processing, which explains their optimal activity

at acidic pH. The cysteine cathepsins are synthesized as inactive zymogens and are activated upon proteolytic removal of the N-terminal propeptide. Although initial studies dictated an intracellular role for cathepsins, recent data demonstrated that some of them, cathepsins B, K and L, can be secreted and positioned on the cell membrane. Localized extracellular, cathepsins have been implicated in cell invasion, interstitial matrix and bone remodeling, tumor growth, antigen processing by T cells, and apoptosis. Among the potential extracellular roles for cysteine cathepsins are cleavage of extracellular matrix proteins and adhesion molecules and activation of enzymes such as urokinase-type plasminogen activator (uPA). Their intracellular activity is regulated by steffins. Extracellular cysteine cathepsins are inhibited by cystatins and kininogens. The latter are large extracellular proteins that contain three cystatin-like domains, two of which exhibit inhibitory activity[64].

Although it has been established that cathepsin function can be split up into intracellular terminal protein processing and extracellular matrix degradation, Szpaderska *et al*. have identified a novel pathway of cross-talk between the endolysosomal system and the cellular micro-environment. They demonstrated that invasiveness of human melanoma and prostate carcinoma cells was only reduced by intracellular inhibition of cathepsin B[65]. Similarly, type IV collagen degradation by human breast carcinoma cells was only diminished by cell membrane-permeant inhibitors[66]. The proposed mechanism is that uPARAP (uPA receptor-associated protein) links specific cathepsin(s) to uPA/uPAR. uPARAP forms a trimolecular complex with pro-uPA and uPAR, which is essential for the cellular uptake of collagen and subsequent degradation in lysosomes by cathepsins[5]. In addition, cysteine cathepsins can initiate uPAR associated cell-surface proteolysis through activation of uPAR bound pro-uPA to uPA with subsequent plasmin generation[5].

EXOPROTEASES: AMINO- AND CARBOXY-PEPTIDASES

Amino- and carboxy-peptidases are exopeptidases, which clip off one or two of the N- and C-terminal amino acids from proteins and pepides for maturation, activation or degradation, and thereby relate to a variety of biological processes. They belong to the family of metalloproteinases that remove amino acids from unblocked N-termini. For enzymatic activity a conserved amino acid sequence is used as a scaffold to bind one or two divalent metal ions such as Mn^{++}, Zn^{++} and Co^{++}. With respect to localization, some of these exopeptidases are secreted, while others are membrane-bound or cytosolic.

Three aminopeptidases have been implicated in angiogenesis, knowing 2 methionine aminopeptidase, aminopeptidase N (CD13)[67, 68], and adipocyte-derived leucine aminopeptidase, also called puromycine insensitive leucyl-specific aminopeptidase

(PILSAP)[69]. Methionine aminopeptidase 2 was shown to be a target for the anti-angiogenic molecules fumagillin and ovalicin[70, 71]. CD13 is exclusively expressed in endothelial cells (EC) of developing vessels and not in the normal quiescent vasculature[72]. This recognition has prompted studies on using CD13 as a target for inhibiting tumor vascularization[72, 73] and on its role in angiogenesis[67, 68]. Sato *et al*.[69] suggested that PILSAP is involved in growth and differentiation of vascular and hematopoietic populations from those precursors. The potent angiogenic factor VEGF induced PILSAP expression in endothelial cells *in vitro*, and was localized to sites of angiogenesis *in vivo*. Furthermore, specific elimination of PILSAP expression by siRNA interference abrogated VEGF induced EC proliferation, migration and network formation *in vitro*, and angiogenesis *in vivo*.

Carboxypeptidase N, also called TAFI, can indirectly affect angiogenesis by limiting fibrinolysis and modifying SDF-1[74].

MATRIX PROTEOLYSIS AND ANGIOGENESIS

When endothelial cells form a capillary sprout they must invade the tissue and create space for expansion of the new vessels to be formed. It is obvious that involvement of proteases in angiogenesis initially focused on the degradation of the extracellular matrix. This encompasses first the degradation of the endothelial basement membrane to enable endothelial and accessory cells to migrate into the area of neovascularization, and subsequently proteolysis of components of the interstitial matrix to create space for a vascular lumen. Although each member has its own specific substrate specificity, matrix-degrading metalloproteinases (MMP) in concert are able to degrade a wide if not the whole spectrum of matrix proteins. Quiescent endothelial cells produce little or no MMP, whereas these molecules are strongly upregulated in activated endothelial cells *in vitro*[75, 76], and in the endothelium of vessels in wound healing, inflammation and tumors[30, 31, 77]. Therefore MMP are considered to be the prime class of proteases involved in matrix degradation accompanying angiogenesis.

Other proteases, such as plasmin and cathepsin-B, can co-operate with MMP. Once activated, plasmin can act by itself on matrix proteins and is also able to activate various MMP, including MMP-1, -2, -3 and -9[78, 79].

A number of studies including gene deletions in mice have pointed to the essential role of particularly MMP-2, MMP-9 and MT1-MMP in the onset of angiogenesis in tumors and in development and bone formation[80-84]. Mice lacking MMP-2, MMP-9 or MT1-MMP display disturbances in growth and bone formation and impaired angiogenesis. Although the involvement in the onset of angiogenesis, the so-called angiogenic switch[85], may suggest that they stimulate angiogenesis primarily by matrix

degradation, it should be noted that the activities of these proteases are complex and may include other effects as well, such as the activation of growth factors and cytokines, the recruitment of endothelial progenitor cells, and the degradation of inhibitors. This is underlined by the observation that after the onset of tumor angiogenesis MMP-9 also generates angiogenesis inhibitors, such as tumstatin, by which angiogenesis becomes then retarded[86].

PROTEASES CAN ALTER THE BIOLOGICAL ACTIVITIES OF GROWTH FACTORS

The perspective of proteases in angiogenesis has widened by the recognition that, in addition to matrix proteins, growth factors and receptors are also important targets. Indeed, proteases can control the onset and progression of angiogenesis by the activation and liberation of growth factors, as well as the modification of the biological properties of angiogenic growth factors and cytokines[2, 34]. Hepatocyte growth factor (HGF) activating factor, a serine protease related to plasmin, converts HGF into an active angiogenesis factor[87]. Growth factors with heparin binding properties, such as bFGF and VEGF, can be liberated from matrix proteoglycans by heparinases and proteases[88, 89]. VEGF can be set free into an active state from the connective tissue growth factor (CTGF)/VEGF complex by MT-MMP-mediated proteolytic cleavage of CTGF[90]. Similarly, growth factors that indirectly affect angiogenesis can be targets of proteolytic activation, *e.g.* latent TGF-β can be activated by the action of plasminogen activators and thus becomes involved in angiogenesis in bone[91, 92].

Proteolysis not only contributes to the liberation or activation of growth factors, but also can alter their functional properties. This was elegantly shown for $VEGF_{165}$, which upon cleavage by either MMP-3 or MMP-9 is reduced to a smaller molecule with properties similar to $VEGF_{121}$[93]. In contrast to $VEGF_{165}$, which induces a regular vessel pattern during neovascularization, the shortened VEGF obtained by MMP-3 or -9 cleavage induced an irregular vessel pattern comparable to $VEGF_{121}$, which may reflect the fact that these molecules do not bind to heparin sulfates[93].

The properties of another important factor in neovascularization, stromal cell-derived factor-1 (SDF-1) are also modified by proteases, but in this case by carboxy- and amino-terminal truncations. The two isoforms of SDF-1, SDF-1a and SDF-1b, are both modified by the aminodipeptidase DPPIV/CD26, by which their heparan sulfate affinities and interactions with their receptor CXCR4 are reduced[94]. SDF-1a is additionally shortened by 1 amino acid by carboxypeptidase N[74], which further reduces the affinity for heparan sulfates. MT1-MMP, MMP-1, -2 and -13 can cleave and inactivate SDF-1 and its receptor CXCR4[95]. VEGF and SDF-1 often participate simultaneously in angiogenesis, particularly when bone marrow recruited progenitor

cells are involved[96].

Activation of Notch-1 signaling, which has now been well recognized to play a role in endothelial differentiation and embryonic and tumor angiogenesis[38, 39], occurs by specific cleavage of the Notch receptor after ligand binding. The aspartate proteinase presenilin is the active protease in the g-secretase complex that cleaves Notch-1 upon ligand binding[97]. Furthermore, both ADAM-17 and ADAM-10 may contribute to Notch signaling by performing a specific cleavage essential for Notch receptor activation upon ligand binding, or by shedding the Notch ligand Delta from the cell surface[36, 37]. ADAM-10 was also shown to generate cleaved soluble Eph receptors by shedding of EphA2 and EphA3[98, 99], which can inhibit tumor angiogenesis in mice[100].

MIGRATION AND INVASION DEPEND ON PERICELLULAR PROTEOLYSIS

Migration and invasive growth of cells involved in angiogenesis requires a delicately balanced interplay between detachment and new formation of cell adhesions to enable the cell to crawl forward through the extracellular matrix[10]. To this end, the cell generates limited proteolytic activity at individual focal adhesions often via the formation of multiprotein complexes. Indeed, in recent years it has become clear that multiprotein complexes are built up in lipid rafts on the cell surface, and those membrane-bound proteases often take part therein. For example in invading leukocytes, a complex containing u-PA, uPAR and integrins has been recognized that participates in cell adhesion and invasion. uPAR acts here as an organizing center being able to form non-covalent complexes with integrins, LRP-like proteins and u-PA or uPA:PAI-1. Such complexes also occur on endothelial cells (compare Fig. 1). Other membrane-associated metalloproteinases, such as MT-MMP, can participate in similar multiprotein complexes. Analogous to the interaction of integrins with uPA/uPAR, the localization of MMP-2 on the cell membrane can be associated with $\alpha_V\beta_3$-integrin, which aids in focusing proteolytic activity[81, 101]. MT1-MMP co-localizes with β1-integrins in cell-cell contacts, whereas it was encountered with αvβ3-integrins in migrating endothelial cells[102]. MMP-9 interacts with the cell adhesion molecule CD44[103], which on its turn is processed by MT1-MMP[104]. Furthermore, MMP-2 binds, in a complex reaction with TIMP-2, to MT1-MMP on the cell surface[33]. This interaction facilitates the activation of MMP-2 by a second adjacent MT1-MMP molecule.

PERICELLULAR ACTIVITIES OF MT1-MMP

The control of pericellular proteolytic activity involved in cell migration can be demonstrated by the action of MT1-MMP. After intracellular activation by furin-like proteases MT1-MMP is inserted in the plasma membrane. It is directed towards the lamellipodia at the front of migrating cells, which suggests an interaction between MT1-MMP and the actin cytoskeleton[104]. This interaction facilitates the dimerization of MT1-MMP molecules, which is required for the activation of MMP-2 as mentioned above. Once activated, both MMP-2 and MT1-MMP can degrade various proteins of the extracellular matrix as well as several membrane proteins including a_v-integrins and CD44[104, 105]. Subsequently, MT1-MMP is internalized and either degraded or recycled to the plasma membrane, depending on additional regulation[106-108]. In other cell types it was shown that the internalization of MT1-MMP was needed for cell migration[109]. Thus, it is probable that treadmilling of MT1-MMP in complex with adjacent (receptor) proteins is required for the locomotion of the cell. MT1-MMP-mediated migration of endothelial cells in a collagen substrate/matrix indeed depends on caveloar internalization of MT1-MMP[108]. Other membrane spanning MT-MMP may substitute for MT1-MMP in specific conditions, such as MT3-MMP in human endometrial microvascualr endothelial cells[110].

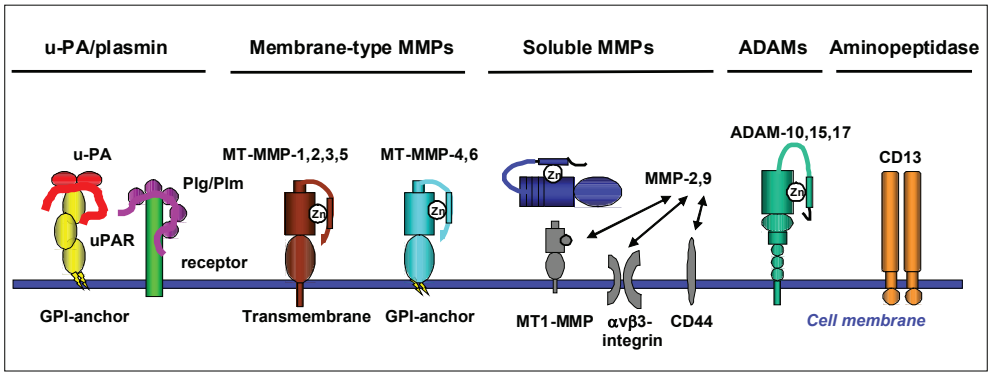

Fig 1. Various types of membrane-associated proteases

Various membrane-associated proteases participate in the regulation of angiogenesis. These proteases are anchored to the membrane by a transmembrane domain (MT-MMP1, ADAMs, CD13), a GPI-anchor (uPAR, MT-MMP4) or by interaction with other membrane-bound proteins (CD44 for MMP-9; MT1-MMP and □v□3-integrin for MMP-2). This interaction regulates the localized action of proteases on and shuttling over the cell surface, and can participate in the subsequent internalization, which are all needed for cell movement and invasion (see text).

Abbreviations: u-PA: urokinase-type plasminogen activator; uPAR: u-PA receptor; Plg/Plm: plasminogen/plasmin; MMP: matrix metalloproteinase; MT-MMP membrane-type-MMP, ADAM: proteases with a disintegrin and metalloproteinase domain. GPI-anchor: glycosylphosphatidylinositol-anchor.

PERICELLULAR PROTEOLYSIS BY u-PA/uPAR AND MMP

MT1-MMP and u-PA/uPAR both display a comparable treadmilling mechanism at the front of the migrating cell (compare[10, 32]). Prager *et al*.[111] showed that uPAR is redistributed to focal adhesions at the leading edge of endothelial cells in response to VEGF, and that subsequent induction of cell migration depends on u-PA activation, interaction of u-PA with PAI-1 and internalization of this complex bound to uPAR. VEGF-dependent activation of the uPAR-bound u-PA involves a change in integrin affinity and MMP-2 activity bound to MT1-MMP on these cells. Other investigators reported on MT-MMP and u-PA/plasmin as additionally acting mechanisms with a mutual balance between these proteases depending on the matrix conditions[112, 113]. Many data on the role of cell-bound u-PA and plasmin on endothelial cell migration and tube formation have been obtained in *in vitro* studies[114-116]. However, the evidence for the involvement of this system is angiogenesis *in vivo* is less generally accepted. Normal blood vessels develop in animals deficient of plasminogen, but neovascularization is disturbed in VEGF- and bFGF-induced angiogenesis in the cornea of these mice; data on u-PA-deficient animals are unequivocal[117, 118]. Neovascularization after myocardial infarction depends equally on u-PA/plasmin activities as on MMP[119]. Similarly, neovascularization was reduced by 50% in the fibrinous exudates of plasminogen deficient mice (Collen and van Hinsbergh, unpublished). On the basis of the data available one cannot discriminate yet whether the u-PA/plasmin contribution acts largely via endothelial cells on angiogenesis or that the invasion of leukocytes and endothelial progenitor cells, which may supply additional growth factors, also contributes to this effect (see below).

EXCESSIVE PROTEOLYSIS INHIBITS NEOVASCULARIZATION

The proteolytic activities involved in matrix destruction and remodeling require spatial and temporal control. Excessive proteolysis can cause unwanted damage to the tissue and might dissolve the matrix needed for anchoring the migrating cells. This was elegantly shown in mice deficient for plasminogen activator type-1 (PAI-1)[120, 121]. Because PAI-1 inhibits plasminogen activators and hence plasmin activation, one would expect that PAI-1 deficiency would increase angiogenesis and tumor growth. However, when PAI-1 deficient mice were challenged with xenografted cancer cells on a collagenous matrix, angiogenesis and vascular stabilization were severely impaired, thereby hampering tumor growth. Indeed, PAI-1 protects the surrounding extracellular matrix from excessive degradation by plasmin, thus maintaining a foothold for the endothelial cells that migrate and form capillary structures to nourish the tumor[121-123]. Improper proteolytic processing also underlies the disrupted vascular development and premature deaths of murine embryos deficient of the inhibitor

RECK. This is likely due to uncontroled MMP activity, because a partial rescue was obtained in mice that were deficient for both RECK and MMP-2[124].

PROTEASES CONTROLLING ANGIOGENESIS IN A FIBRIN MATRIX

A specific condition in angiogenesis associated with wound repair and pathological conditions is the neovascularization of a fibrinous matrix. Fibrin acts as a temporary matrix, which not only prevents loss of blood, but also facilitates the subsequent healing process, including angiogenesis needed for the formation of granulation tissue. Plasmin and its plasminogen activators, probably in concert with MMP, play a role in the neovascularization of fibrinous exudates, as occurs in healing wounds, infarcted myocardium and tumor stroma[119, 125, 126]. In addition to plasmin, MT1-MMP can act as a fibrinolysin and facilitate capillary outgrowth in a fibrinous environment[127, 128]. In a fibrin matrix both plasmin and MT1-MMP have been indicated to be involved in endothelial tubule formation[112, 113, 125, 127, 129]. In endothelial cells in vitro both tubular outgrowth and MT1-MMP activity were inhibited by TIMP-2 and-3 but not by TIMP-1[112, 113, 129]. Interestingly, tube formation by endothelial cells of human endometrium was equally inhibited by TIMP-1 and TIMP-3 and may depend on MT3-MMP activity[110]. Indeed, overexpression of MT1-, MT2- or MT3-MMP, but not MT4-MMP, each enhances the autonomous fibrin-invasive activity of endothelial cells[128].

CYSTEINE CATHEPSINS AND ANGIOGENESIS

A number of studies show the presence of cathepsins in the vasculature of human tumors associated with an increase in angiogenesis and tumor growth[130, 131]. Inhibition of cathepsin activity by a broad-spectrum cysteine cathepin inhibitor reduced tumor vascularization and vascular branching during pancreatic islet tumorigenesis in RIP1-Tag2 mice[132]. Remarkably the core of the tumors was more affected than the outer part, possibly related to co-option of existing vessels in the outer region of the tumor. In particular, the cathepsins B, H, L, S and X/Z were expressed in the tumor cells, infiltrating leukocytes and the endothelial cells of the tumor[132]. Subsequently, Gocheva *et al*.[133] showed that mice deficient of cathepsins B or S displayed impaired tumor formation and angiogenesis, while cathepsin B or L knockouts had retarded cell proliferation and tumor growth.

Cathepsin B may also affect endothelial tube formation via intracellular proteolytic activity[134]. Cathepsin B also binds to the cell surface via the light chain of the annexin II heterotetramer and the therewith-associated protein p11, where it becomes active[135]. Cathepsin B has been indicated to enhance cell migration and angiogenesis by activation of uPAR-bound u-PA[136]. Rao and colleagues have shown that simultaneous inhibition of cathepsin B and uPAR by siRNAs markedly reduced the growth

of gliomas and their vasculature in mice[137]. Another mechanism, by which cathepsin B may enhance angiogenesis, may be the degradation of TIMP-1 and TIMP-2, which will result in increased MMP activities[138].

Wang *et al*.[139] suggested that cathepsin S controls angiogenesis and tumor growth via matrix-derived angiogenic factors. Null mutants of cathepsin S also showed an impaired development of microvessels during wound repair[140]. Furthermore, cathepsin S facilitated *in vitro* endothelial tube formation and invasion in Matrigel or a collagen 1 matrix[140]. Angiogenesis is generally thought to be enforced by so-called endothelial progenitor cells. These cells express a range of cathepsins, of which cathepsin L was shown to contribute to angiogenesis in mice (see below)[141]. These data strengthen the idea that cathepsins indeed play a role in angiogenesis in tumors and tissue repair.

PROTEASES AND THE RECRUITMENT OF BONE MARROW DERIVED CELLS

Leukocytes and endothelial progenitor cells can contribute to the initiation and guidance of new blood vessels[142, 143]. u-PA/uPAR and MT1-MMP play a role in monocyte recruitment during inflammation[144, 145], as also MMP-9 does. Monocytes produce various pro-angiogenic factors[142]. Furthermore, a special population of $CD34^+$ cells which can acquire endothelial-like properties, such as the expression of VE-cadherin and VEGF receptor-2 (*kdr, flk-1*) are thought to markedly influence the progression of angiogenesis[143, 146]. Their absence or dysfunction is associated with impaired vascularization in cardiac and diabetes patients[147, 148]. For a discussion of the types of circulating endothelial/progenitor cells the reader is referred to Ingram *et al*.[149] Proteases play a role both in the mobilization of hemapoietic and endothelial progenitor cells in the bone marrow and in the recruitment of these cells into the areas of starting and ongoing neovascularization.

MMP-9 AND MOBILIZATION OF ENDOTHELIAL PROGENITOR CELLS

MMP-9 received special attention in the mobilization of various types of progenitor cells (Fig. 2). The bone marrow is divided into an osteoblastic and a vascular zone. Within the osteoblastic zone most stem cells are in contact with stromal cells, retaining them in the G_o phase of cell cyle, whereas in the vascular zone a small fraction of the stem cells is in the S or G_2/M phase. This equilibrium between these two compartments is dictated by the bioavailability of specific stem cell-active cytokines, such as KitL, (stem cell factor) which are either stromal cell- or matrix bound. Heissig *et al*.[150] studied MMP-$9^{-/-}$ mice treated with 5-fluorouracil (5-FU), a cell cycle cytotoxic agent, which causes bone marrow ablation. Myelosuppression by 5-FU resulted in elevated VEGF and SDF-1 plasma levels, which promoted MMP-9 expression. Sub-

sequently, active MMP-9, produced by bone marrow stromal cells, causes shedding of soluble Kit ligand (sKitL).

Hematopoietic stem cells, cardiac, epithelial and endothelial progenitor cells all express the receptor for sKitL, c-Kit[151, 152], and upon binding migrate towards the vascular zone where they proliferate and differentiate, and finally enter the circulation. MMP-9 $^{-/-}$ mice displayed an impaired sKitL release, which coincided with a delayed HSC motility, resulting in failure of hematopoietic recovery and increased mortality. Exogenous sKitl administration restored hematopoiesis and survival after bone marrow ablation. Similar experiments performed in Nos3 $^{-/-}$ mice demonstrated that NO functioned as a paracrine factor in the VEGF-induced MMP-9 activation[153]. Endothelial cells, which constitute a significant part of the stroma, react upon VEGF activation by Akt-dependent eNOS phosphorylation. Recent data identified MMP-9 as a major target for NO, via S-nitrosylation of MMP-9[154]. The release of sKitL by VEGF-induced MMP-9 mobilizes endothelial and mast progenitor cells in a comparable way[155]. Mast cell precursors migrate towards an ischemic tissue, where they mature and start to produce VEGF. VEGF creates a positive feedback loop to the bone marrow where it activates MMP-9, finally leading to further recruitment of progenitor cells.

PROTEASES AND THE RECRUITMENT OF EPC TO THE ANGIOGENESIS AREA

Next to its role in stem cell mobilization, various reports describe the expression of MMP-2 and MMP-9 in peripheral blood and bone marrow CD34^{+} progenitors as well as cultured EPC. Stimulation with TNF, IL-8 or SDF resulted in increased MMP levels, facilitating the migration of EPC into Matrigel plugs or transwell systems[156-159]. Not only MMP are involved in EPC-enhanced angiogenesis. Endothelial progenitor cell-derived cells (EPDC) displayed higher levels of uPA and uPAR compared to normal endothelial cells. Inhibition of EPDC-associated uPA by monoclonal antibodies that block uPA activity or receptor binding, significantly reduced proliferation, migration and capillary like tube formation[160]. Selleri *et al*.[161] postulated a novel concept for the uPA/uPAR system in stem cell mobilization. Cleaved forms of soluble urokinase receptor (c-suPAR), derived from comobilized myeloid and monocytic cells, were identified in healthy patients during granulocyte colony stimulating factor (G-CSF)-induced mobilization of CD34^{+} HSC as well as in sera of patients affected by various tumors. *In vitro*, c-suPAR was able to chemoattract HSC by activating the high-affinity fMet-Leu-Phe (fMLP) receptor (FPR). In addition, the suPAR-derived peptide uPAR(84-95) inhibited migration of HSC towards stromal cell-derived factor (SDF-1). *In vivo*, uPAR(84-95) increased the number of HSC in peripheral blood, and inactivated murine CXCR4, through an unknown mechanism[162]. Since CXCR4 is known as a retention factor for stem cells in the bone marrow as well as in peripheral tissue,

inactivation by c-suPAR might explain this mobilizing effect[96, 163].

Since endothelial progenitor cells promote neovascularization after ischemia, while mature endothelial cells do not display this feature, several groups have compared these two distinct cell populations. Urbich *et al*.[141] compared gene expression profiles of cultured blood-derived endothelial progenitor cells with human umbilical vein endothelial cells (HUVEC) and $CD14^+$ monocytes. Lysosomal cysteine peptidases (cathepsins) were among the most consistent classes expressed differentially. After confirmation on protein level and activity, they focused their attention on cathepsin L, which is also important for tumor invasion and metastasis[164]. Cathepsin L is able to exert its extracellular proteolytic activity via binding of an amino acid segment of the p41 splice variant (p41(65aa)) of major histocompatibility complex class II-associated invariant chain. This complex is able to accumulate and remain active extracellular even at neutral pH[165]. Not only evidence was obtained for the positive role of EPC in improving neovascularization, this effect was dominantly regulated by cathepsin L[141]. Recovery from hind limb ischemia was substantially impaired in cathepsin $L^{-/-}$

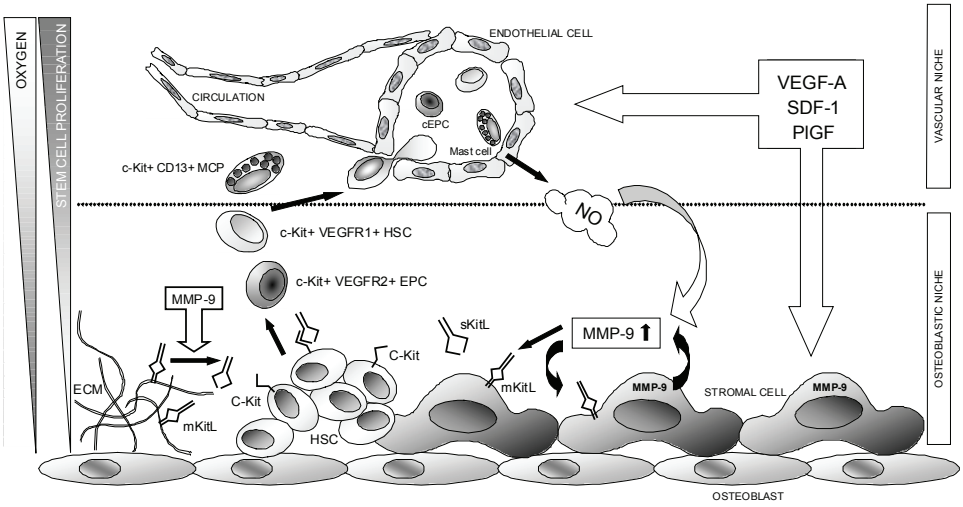

Fig 2. Mobilization of bone marrow-derived progenitor cells

Vascular trauma results in the plasma elevation of angiogenic factors, including VEGF-A, SDF-1 and PlGF. These factors signal to stromal cells in the osteoblastic zone of the bone marrow, resulting in MMP-9 secretion. This process is further enhanced by bone marrow endothelial cell-produced nitric oxide (NO). Active MMP-9 converts membrane- or extracellular matrix-bound mKitL into soluble KitL (sKitL). sKitL binding to the receptor c-Kit on hematopoeitic stem cells (HSC), enhances the cycling, differentiation and mobilization of endothelial progenitor cells (EPC), mast cell progenitors (MCP) and HSC. These cells leave their hypoxic environment, which maintains their 'stemcell-ness', and migrate towards the vascular zone of the bone marrow where they enter the circulation. Within the circulation these cells mature and are capable of homing towards sites of neovascularization. Within the angiogenic area mast cells create a positive feedback loop by producing angiogenic factors; signalling back to the bone marrow.

mice. Because Urbich *et al*.[141] showed that pharmacological cathepsin L inhibitors and genetic ablation of cathepsin L did not affect the angiogenic activity of mature endothelial cells; a role for cathepsin L in classical sprouting angiogenesis seemed unlikely. In contrast, inhibition of cathepsin L interfered with EPC invasion and incorporation in tumor capillaries *in vivo* and reduced the incorporation and functional activity of EPC in the hind limb ischemia model, resulting in less improvement of limb perfusion. Similar results were obtained when bone marrow derived $Lin^{-}Sca\text{-}1^{+}$ progenitors, representing more immature progenitor cells, were studied.

ENDOGENOUS INHIBITORS GENERATED BY PROTEASES: MATRIKINES LIMITING ANGIOGENESIS

Invasive growth and angiogenesis are accompanied by proteolytic degradation of matrix proteins. Interestingly, among the proteolytic degradation products derived from extracellular matrix proteins and hemostasis factors a number of fragments have potent angiogenesis inhibiting properties. After the initial recognition of a thrombospondin fragment in 1990[166, 167], Folkman and co-workers - in a search for primary tumor factors that suppressed the growth of metastases - isolated angiostatin and endostatin from the urine and tumor tissue of mice that had received grafts of tumors[168, 169]. At present a whole range of angiogenesis inhibiting (and some stimulating) fragments of matrix proteins and coagulation related proteins have been recognized, such as angiostatin, endostatin, tumstatin, alphastatin, thrombospondin fragments and many others. These fragments are indicated as matrikines and have been the subject of various recent reviews, to which the reader is referred[34, 170, 171].

PERICYTE PROTEASES AND STABILIZATION 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[172]. Without pericyte covering, the immature vessels remain dependent on the continuous exposure to angiogenic growth factors, particularly VEGF[172]. The endothelial cells become apoptotic, when the supply of angiogenic growth factors cedes[173, 174].

MMP play a role in the recruitment of pericytes[175]. Animals deficient of MMP-9 had an impaired pericyte mobilization, which markedly affected the extent and stability of neovascularization of neuroblastomas[175]. MMP-9 was found in pericytes present in the stroma of human breast tumors[176]. Aminopeptidase A was also present in activated pericytes in various pathological conditions associated with angiogenesis, while its expression was low in pericytes of quiescent vessels[177], but it is not yet known whether this protease is causally involved in angiogenesis. Pericyte-derived TIMP-2 and TIMP-3 can inhibit MT1-MMP dependent MMP-2 activation on the endothelial

cells, and thus may contribute to the stabilization of newly formed microvessels[112, 178]. Apparently, the balance between proteolytic activity and proteinase inhibition is important. *In vitro* EC-pericyte interactions strongly induce TIMP-3 expression by pericytes, whereas EC produce TIMP-2 in EC-pericyte cocultures[179]. While MT1-MMP is necessary for endothelial tube formation, the suppression of EC TIMP-2 and pericyte TIMP-3 expression leads to capillary tube regression in these cocultures in an MMP-1-, MMP-10-, and ADAM-15-dependent manner[179].

PERSPECTIVE

From the foregoing it is clear that proteases have a complex role in neovascularization, a role that extends to various aspects of growth, wound healing and tissue repair. Multiple targets for individual proteases and overlap of activities of different proteases cause that proteases can be pro- and anti-angiogenic depending on the local conditions. Notwithstanding this complexity, it is likely that specific proteases can be diagnostic or prognostic determinants for specific tumors or the treatment of a disease. For example the recognition of cathepsin K in giant cell tumors of bone, which is active in conjunction of a proton pump[180], improves insight in the osteolysis and provides new therapeutic opportunities. The highest expectations for therapeutic application of inhibiting proteases have been in the field of MMP.

Indeed, for several decades MMP have been heralded as promising targets for cancer therapy, based op their massive upregulation in malignant tissue, and their involvement in matrix destruction, cell invasion, and angiogenesis[181]. Therapeutic strategy of controlling cancer by broadly targeting MMP inhibitors (MPI) was founded on reducing the degradation of basement membrane and extracellular matrix proteins by cancer cells in metastasis and angiogenesis. Although promising results were obtained in animal models for cancer progression and metastasis, results from phase III clinical trials were disappointing (see Cousssens *et al*. for overview[182]). Several trials have been terminated for lack of efficacy. The premature termination of studies using tanomastat was of even greater concern, as patients receiving MPI showed significantly poorer survival than the placebo group[183]. MMP inhibition, in some cases, resulted in increased number of liver micrometastases, less differentiation of tumors (representative of a more aggressive phenotype), and reduced production of the angiogenesis inhibiting peptide angiostatin[182, 184]. Today, it is clear that the major role of MMP is for homeostatic regulation of the extracellular environment, modification of proteins and for controlling innate immunity[2, 181], not simply to degrade extracellular matrix.

Although previous generations of MMP inhibitors failed, new more selective inhibitors are developed. For successful cancer treatment based on MMP inhibition, the

next generation of MMP inhibitors drugs must be selective against validated MMP targets without affecting molecules that cause worsening of the disease or serious side effects when no longer degraded or modified by the MMP to be inhibited.
Recognition of the complexity of action of proteases such as MMP also provides new opportunities for more specific treatment. In particular the recognition of matrix degradation products that inhibit angiogenesis, matrikines[170], provide a spectrum of new approaches how to reduce unwanted neovascularization. Furthermore, better understanding the cellular targets of these matrikines further provide new leads for influencing angiogenesis, both by inhibition in cancer, rheumatoid arthritis and other diseases, as well as by enhancing angiogenesis in poorly healing wounds and ischemic heart tissue.

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