Pericellular Proteases in Angiogenesis and Vasculogenesis

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Abstract—Pericellular proteases play an important role in angiogenesis and vasculogenesis. They comprise (membrane-type) matrix metalloproteinases [(MT-)MMPs], serine proteases, cysteine cathepsins, and membrane-bound aminopeptidases. Specific inhibitors regulate them. Major roles in initiating angiogenesis have been attributed to MT1-matrix metalloproteinase (MMP), MMP-2, and MMP-9. Whereas MT-MMPs are membrane-bound by nature, MMP-2 and MMP-9 can localize to the membrane by binding to αvβ3-integrin and CD44, respectively. Proteases switch on neovascularization by activation, liberation, and modification of angiogenic growth factors and degradation of the endothelial and interstitial matrix. They also modify the properties of angiogenic growth factors and cytokines. Neovascularization requires cell migration, which depends on the assembly of protease–protein complexes at the migrating cell front. MT1-MMP and urokinase (u-PA) form multiprotein complexes in the lamellipodia and focal adhesions of migrating cells, facilitating proteolysis and sufficient support for endothelial cell migration and survival. Excessive proteolysis causes loss of endothelial cell-matrix interaction and impairs angiogenesis. MMP-9 and cathepsin L stimulate the recruitment and action of blood- or bone-marrow-derived accessory cells that enhance angiogenesis. Proteases also generate fragments of extracellular matrix and hemostasis factors that have anti-angiogenic properties. Understanding the complexity of protease activities in angiogenesis contributes to recognizing new targets for stimulation or inhibition of neovascularization in disease. (Arterioscler Thromb Vasc Biol. 2006;26:716-728.)

Key Words: endothelium ■ neovascularization ■ matrix metalloproteinases ■ MT-MMPs ■ cathepsins
associated with angiogenesis. These split products have an important effect on the delicate balance between factors that enhance cell migration and growth and factors that keep the endothelial cells in a quiescent and more differentiated state, and can shift this balance toward the onset and progression of angiogenesis.

Proteases that are involved in angiogenesis can act intracellularly or extracellularly. Among the intracellular proteases several are involved in protein maturation and processing, such as furin. Furthermore, proteasome-related proteases degrade not only deformed proteins but also are involved in controlling the survival of transcription factors, such as hypoxia-induced factor-1α (HIF-1α), that regulates expression of angiogenesis factors. Lysosomal proteases control degradation of internalized proteins and also digest extracellular matrix proteins that are ingested in endosomal bodies. Caspases, a group of cysteinyl aspartate-specific proteases, are key regulators of apoptosis, a process pivotal in endothelial death and vascular pruning. Extracellular proteases are involved in the degradation of matrix proteins. On the basis of devastating diseases such as cancer, rheumatoid and bone diseases the focus has been initially on destruction of matrix mass in these tissues and during invasive growth. We also recognize a more delicate pericellular degradation of matrix proteins and cellular receptors during the progression of cell migration and invasion. Furthermore, well-coordinated pericellular proteolytic activities control the availability of active and modified angiogenic growth factors and cytokines.

In the present survey, we focus on pericellular proteolytic activities involved in angiogenesis and vasculogenesis. This comprises pericellular proteolysis involved in cell migration and invasion of endothelial cells and cells that support the formation of new and stable microvessels; the activation and modification of growth factors; and the generation of new epitopes in tailored proteins and protein fragments with new angiogenic and anti-angiogenic properties. After a short survey of the major classes of proteases involved, we discuss these proteases according to the biological effects that they perform during neovascularization.

Types of Proteases Involved in Matrix Degradation and Angiogenesis

Three major groups of endoproteases play an important role in the processes that regulate angiogenesis, including the remodeling of the extracellular matrix, cell migration, and invasion, and the liberation and modification of growth factors. They comprise the metalloproteinases, in particular the matrix metalloproteinases (MMPs), the cathepsin cysteine proteases and the serine proteases. The activities of these proteases are controlled by specific activation mechanisms and specific inhibitors, of which tissue inhibitors of metalloproteinases (TIMPs), cystatins, and inhibitors of serine proteinases called serpins represent major classes. Many of these proteases act on the cell surface either because they contain a membrane spanning or binding domain or as the result of their interaction with specific receptors on the cell surface. In addition, inhibitor studies with fumagillin derivatives have pointed to a role of aminopeptidases in angiogenesis.

Metalloproteinases: MMPs, ADAMs and ADAMTSs

MMPs belong to a family of zinc-dependent endopeptidases that digest specific extracellular matrix components, and also are able to aid in the activation of proteins such as other MMPs and growth factors. These enzymes are important for cell migration and invasion, and regulate developmental growth and many remodeling processes, such as angiogenesis, arterial remodeling and wound healing. The MMPs have a specific structure bearing several homologous domains as depicted in Figure 1. They can be arranged in 2 groups, as soluble MMPs and membrane-type MMPs (MT-MMPs).

The soluble MMPs are expressed as inactive pro-enzymes that only become activated once they are present in the extracellular environment. This family of MMPs harbors collagenases (MMP-1, MMP-8, and MMP-13), gelatinases...
glutaminase.17–19 In this context it is of interest that CD44 and laminin-5, aggrecan and fibronectin, and the plasma-derived matrix components such as fibrillar collagen, laminin-1, and (GPI) anchor (MT4/6-MMP). The MT-MMP family members were first identified as activators of soluble MMPs, and subsequently also shown to be able to degrade extracellular matrix components such as fibrillar collagen, laminin-1, and laminin-5, aggrecan and fibronectin, and the plasma-derived matrix proteins vitronectin, and fibrin.11,13,15 Furthermore, they can proteolytically liberate and modify growth factors and cytokines.4

Importantly, MT-MMPs at the cell surface pinpoint MMP-dependent matrix degradation to the close vicinity of the cell. MT1-MMP can also locally activate the proforms of MMP-2 and MMP-13, which focuses proteolytic activity on specific sites on the cell surface that are involved in cell migration and invasion.11,16 Furthermore, MT1-MMP processes cell adhesion molecules CD44 and αvβ3-integrin and tissue transglutaminase.17–19 In this context it is of interest that CD44 and αvβ3-integrin have been recognized as binding moieties for MMP-9 and MMP-2, respectively.20,21

Because of the unexpected effects of broadly acting metalloproteinase inhibitors, much interest was generated to search for other types of metalloproteinases. These searches recognized two additional large families of metalloproteinasem, the ADAM (a disintegrin and metalloproteinase domain) family, which are membrane-spanning proteins, and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family, which are secreted into the interstitial space and bind to extracellular matrix proteins.22,23 ADAMS have a transmembrane domain and can act as sheddases on the surface of the cell. At least 34 ADAMS are known, of which 19 have proteolytic activity.13,22 Three members (ADAM-10, ADAM-15, ADAM-17) are known that have properties directly important for the regulation of angiogenesis, but other members may add, as they have not yet been investigated sufficiently.

The ADAMTS proteins are a family of secreted metalloproteinasem that contain one or several thrombospondin domains, by which many of them bind to extracellular matrix proteins.23 Nineteen human members of the ADAMTS family are known, of which ADAMTS-1, -4, -5, -8, and -15 represent a vetebrate subfamily that originated from gene duplication. These latter proteases are involved in the degradation of the proteoglycans versican and aggrecan, and several members of this family, ADAMTS-1 and ADAMTS-8, have an inhibitory effect on angiogenesis.24,25 ADAMTS-4 was indirectly implicated in angiogenesis, as it was differentially expressed in endothelial cells that formed capillary-like tubular structures in a fibrin matrix.26

Inhibitors of Metalloproteinases

In general, inhibition of MMPs occurs in tissues through specific inhibitors, designated as TIMPs, of which at least 4 members are known. MMPs can also be inhibited by α2-macroglobulin and a membrane-anchored glycoprotein called RECK (reversion-inducing cysteine-rich protein with Kazal motifs).27,28 TIMPs bind activated MMPs to form equimolar complexes. Although most soluble MMPs can be inhibited by all TIMPs, MT-MMPs have a more restricted pattern of TIMP inhibition. TIMP-2 and TIMP-3 efficiently inhibit MT1-MMP, while TIMP-1 is rather ineffective in this respect.16 TIMPs also inhibit proteolytically active ADAMs and ADAMTSs, but this inhibition is usually more selective, ie, a higher selectivity for individual TIMPs is observed.13 TIMP-2 can also abrogate angiogenic factor-induced endothelial cell proliferation in vitro and angiogenesis in vivo, independent of MMP inhibition.29 TIMP-2 can enhance the expression of RECK via Rap1 signaling, which causes an indirect, time-dependent inhibition of endothelial cell migration.30 TIMP-3 is sequestered at the cell surface by association with the glycosaminoglycan chains of proteoglycans, especially heparan sulfate, and may play a role in the regulation of ADAM-17.31 TIMP-3 by itself can block vascular endothelial growth factor (VEGF) binding to its receptor and reduce angiogenesis, independent of MMP inhibition.32

Cysteine Cathepsins

In humans, the cathepsin cysteine proteases or cysteine cathepsins consist of a family of 11 members, which are indicated as cathepsins B, C, F, H, L, K, O, S, V, W, and X/Z.33 In the mouse, 19 cathepsins are known including several additional placentally expressed cathepsins that have no human homologue.34 The cysteine cathepsins are synthesized as inactive zymogens and are activated by proteolytic removal of their N-terminal propeptide. Their activity is regulated intracellularly by a specific cystatin subgroup called stefins and extracellularly by cystatins and kininogens.35

The majority of cysteine cathepsins are endopeptidases.33 Cathepsins are primarily known as lysosomal enzymes, but new data indicate that they also play a role in antigen processing by T cells, in cell invasion, extracellular matrix and bone remodeling, in tumor growth, and in apoptosis.35,36 Cathepsins have their enzymatic activity optimum at acidic pH. This facilitates their activities in lysosomes and endosomal vesicles, in which they are involved in the terminal degradation of proteins. In addition, several cathepsins, particularly cathepsin B, K, and L, are also encountered outside the cells or on the cell membrane,12,37,38 and contribute to angiogenesis and recruitment of endothelial progenitor cells into the angiogenic region. The close association of cathepsin K with a proton pump in the membrane may cause a local pericellular acidic environment that enhances cathepsin activity.37

Serine Proteases

The serine proteases are endopeptidases that have a catalytic site made up by three amino acids, serine, aspartic acid, and histidine, the “catalytic triad.” They represent a vast class of proteases with members with a broad variety of functions. Nearly all members are synthesized as zymogens, which are activated by proteolytic removal of an N-terminal part of the molecule that covers and hides the active side. Although
many serine proteases may indirectly affect proteins involved in angiogenesis, plasminogen activators (PAs) and plasmin received specific attention. For several decades the serine proteases urokinase-type PA (u-PA) and plasmin have been recognized as proteases that are involved in cell migration and invasion.\textsuperscript{39} u-PA can activate the zymogen plasminogen into the broadly acting protease plasmin. This process is facilitated by the u-PA receptor (uPAR).\textsuperscript{40} A second mammalian PA is tissue-type plasminogen activator (t-PA), which activates plasminogen in the presence of fibrin. Its primary function is fibrinolysis in the blood and body cavities, whereas u-PA plays a dominant role in cell invasion. Both u-PA and t-PA are inhibited by plasminogen activator inhibitors (PAIs), in particular PAI-1. Plasmin activity is highly effectively inhibited by \(\alpha_2\)-antiplasmin and also controlled by \(\alpha_2\)-macroglobulin. Although dispensable for development of the vascular tree, plasmin, u-PA and PAI-1 have been reported to modulate angiogenesis in various pathological conditions.\textsuperscript{41,42}

Other serine proteases can also indirectly affect angiogenesis, such as the protein convertases furin and PC6, which are major regulators of the activation of MT-MMPs.\textsuperscript{7} This occurs intracellularly during transport of MT-MMPs to the membrane. Furthermore, several members of the coagulation cascade have an effect on the early development of the vascular system that is independent of their contribution to coagulation.\textsuperscript{43}

Several groups of membrane-bound serine proteases were recently recognized and are expanding rapidly.\textsuperscript{44} They comprise proteins that are bound with a GPI-anchor and proteins that have a transmembrane domain either in their C-terminal part or at their N-terminal part. By their surface-bound nature they may locally modify the biological activities of cytokines and vasoactive agents, as was shown for DPP4/CD26 and the related fibroblast activation peptide-\(\alpha\).\textsuperscript{55–47}

**Aminopeptidases**

Aminopeptidases are exopeptidases that split off 1 or 2 amino acids from the unblocked amino-terminus of proteins. Interest in these proteases was stimulated by the finding that the target for the antiangiogenic compound fumagillin and its derivative TNP-470 was a methionine aminopeptidase-type 2 (MeAP2).\textsuperscript{48,49} Sato\textsuperscript{50} recently reviewed the potential role of this and several other aminopeptidases in angiogenesis, particularly aminopeptidase N (CD13/APN) and puromycin-insensitive leucyl-specific aminopeptidase (PILSAP), also called adipocyte-derived leucine aminopeptidase. PILSAP is expressed in endothelial cells and plays a stimulatory role in postnatal angiogenesis.\textsuperscript{51} Sato\textsuperscript{50} suggested that the effect of fumagillin might be via PILSAP instead via Me-AP2. The availability of new inhibitors that discriminate between these enzymes can elucidate this aspect.\textsuperscript{52}

Aminopeptidase N (CD13/APN) was recognized as being highly expressed on the endothelium of growing vessels\textsuperscript{53} after earlier observations by Saiki et al\textsuperscript{34} that inhibitors of CD13/APN impaired tumor growth. This recognition has prompted studies on using CD13/APN as a target for inhibiting tumor vascularization\textsuperscript{53,55} and on the role of this receptor with protease activity in angiogenesis.\textsuperscript{56,57}

### Switching on Angiogenesis: Matrix Proteolysis

When leukocytes, tumor cells, or endothelial cells invade into a tissue they must pave their way and create space. It is therefore quite understandable that the first studies on the involvement of proteases in these processes regarded the degradation of the extracellular matrix. With respect to angiogenesis this encompasses in particular the degradation of the endothelial basement membrane to enable endothelial and accessory cells to migrate into the area of neovascularization, and proteolysis of matrix components to create space for a vascular lumen. Although each member has its own specific substrate specificity, MMPs in concert are able to degrade a wide if not the whole spectrum of matrix proteins and therefore they are considered to be the prime class of proteases involved in degradation of the endothelial basement membrane and interstitial matrix degradation. Quiescent endothelial cells produce little or no MMPs, whereas the expressions of several MMPs are strongly upregulated in activated endothelial cells in vitro.\textsuperscript{58,59} and in the endothelium of vessels in wound healing, inflammation, and tumors.\textsuperscript{10,12,13} Various reviews have extensively surveyed the substrate specificities of the various MMPs.\textsuperscript{3,10}

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 tumors and in development and bone formation.\textsuperscript{60–64} Although their involvement in the onset of angiogenesis, the so-called angiogenic switch,\textsuperscript{6} may suggest that these MMPs 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 complex role 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.\textsuperscript{65} A recent comparison of the ability to enhance capillary-like tube formation in a collagen-rich matrix indicated that MT1-MMP enables endothelial cells to form invading tubular structures, whereas MMP-2, MMP-9, their cognate cell-surface receptors \(\beta3\)-integrin and CD44, or plasminogen did not.\textsuperscript{66}

With respect to the involvement of other MMPs, it should be noted that redundancies exist, which should be taken in account when interpreting data of gene silencing toward the requirement of these genes in angiogenesis. Furthermore, specific attention should be given in translating data from animals to man. For example, the major interstitial collagenase MMP-1 in humans is absent in mice,\textsuperscript{67} in which its function is largely taken over by MMP-13. Notwithstanding this, MT1-MMP, MMP-9 and MMP-2 can be considered as important regulators of angiogenesis in a broad spectrum of pathological conditions.\textsuperscript{60–64}

Other proteases, such as plasmin and cathepsin-B, can cooperate with MMPs. Once activated, plasmin can act by itself on matrix proteins and is also able to activate various MMPs, including MMP-1,-2,-3, and -9.\textsuperscript{68,69} In pathological conditions, often an additional temporary matrix is formed, which consists primarily of fibrin, but also contains fibronectin and vitronectin. Plasmin and its plasminogen activators, probably in concert with MMPs, play a role in the recanalization of thrombi and in the
neovascularization of fibrous exudates, as occurs in healing wounds, infarcted myocardium and tumor stroma. In addition to plasmin, MT1-MMP can act as a fibrinolytin and facilitate capillary outgrowth in a fibrous environment. In a fibrin matrix both plasmin and MT1-MMP have been indicated to be involved in endothelial tube formation. In endothelial cells in vitro both tubular outgrowth and MT1-MMP activity were inhibited by TIMP-2 and -3 but not by TIMP-1. 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. Overexpression of MT1-, MT2-, or MT3-MMP, but not MT4-MMP, each enhances the fibrin-invasive activity of endothelial cells.

Switching on Angiogenesis: Proteolytic Activation and Modification of Growth Factors

Besides the proteolytic action of proteases on proteins involved in cell–matrix interaction, proteases can control the onset and progression of angiogenesis by the activation and modification of the biological properties of angiogenic growth factors and cytokines. The angiogenesis-inducing growth factor hepatocyte growth factor (HGF) is activated by HGF activating factor, a serine protease related to plasminogen. Growth factors, such as bFGF and VEGF, can become liberated from the extracellular matrix after degradation of proteoglycans. In addition to enhancing the expression of VEGF, MT-MMP can release VEGF from the connective tissue growth factor/VEGF complex by proteolytic cleavage of connective tissue growth factor. Growth factors that indirectly affect angiogenesis are also targets of proteolytic activation. For example, plasminogen activators drive the activation of latent transforming growth factor-β from bone extracellular matrix and thus modulate angiogenesis in bone.

The proteolytic modification of growth factors not only causes their activation but also can modify their properties. This was elegantly shown for VEGF, which on cleavage by either MMP-3 or MMP-9 is reduced to a smaller molecule with properties similar to VEGF. Whereas VEGF induces a regular vessel pattern during neovascularization in tumors, VEGF and the shortened VEGF cleaved by MMP-3 or MMP-9 cause an irregular pattern of neovascularization, probably because these molecules do not bind to heparin sulfates and therefore do not provide spatial information that is buried in the extracellular matrix. The properties of another important factor in angiogenesis, stromal cell derived factor-1 (SDF-1) are also modified by proteases, but in this case by carboxy- and amino-terminal truncations. The 2 isoforms of SDF-1, SDF-1a and SDF-1b, are both are modified by the aminodipeptidase DPPIV/CD26, by which their heparan sulfate affinities and interactions with their receptor CXCR4 are reduced. SF-1a is additionally shortened by 1 amino acid by carboxypeptidase N, which further reduces the affinity for heparan sulfates. MT1-MMP, MMP-1, MMP-2, and MMP-13 can cleave and inactivate SDF-1 and its receptor CXCR4. VEGF and SDF-1 often participate simultaneously in angiogenesis, particularly when bone marrow recruited progenitor cells are involved.

Among the proteases that can alter the balance between pro- and anti-angiogenic factors members of the ADAMS family of proteins are receiving attention. ADAM-17 (tumor necrosis factor [TNF]-converting enzyme [TACE]) proteolytically releases TNFα and HB-EGF from their membrane-bound precursors. These factors can indirectly modulate angiogenesis. Both ADAM-17 and ADAM-10 may contribute to Notch signaling by performing a specific cleavage essential for Notch receptor activation on ligand binding, or by shedding the Notch ligand Delta from the cell surface. This may affect angiogenesis as Notch signaling is involved in endothelial differentiation and in embryonic and tumor angiogenesis. ADAM-10 was also shown to cleave and shed Eph receptors EphA2 and EphA3. Soluble EphA inhibits tumor angiogenesis in mice.

Finally, the proteolytic modification not only regards growth factors and cytokines, but also their receptors. Proteolytic modification of neuropeptide Y by CD26 generated a truncated, neuropeptideY, thereby reducing its affinity for Y1 receptors and increasing that for Y2 receptors. Neuropeptide Y is liberated in ischemic areas and enhanced angiogenesis via Y2/Y5 receptors. mice have reduced angiogenesis in ischemic tissue/muscle.

Excessive Proteolysis: No Grip, No Invasion

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 PAI-1. 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. 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. Improper proteolytic processing also underlies the disrupted vascular development and premature deaths of murine embryos deficient of the inhibitor RECK. This pathology is likely caused by uncontrolled MMP activity, because a partial rescue was obtained in mice that were deficient for both RECK and MMP-2.

Angiogenesis on the Move: Assembling 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. To this end, the cell generates limited proteolytic activity at individual focal adhesions often via the formation of multiprotein complexes. In recent years it has become clear that multiprotein complexes are built up in lipid rafts on the cell surface, and that 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 inva-


The uPAR–Integrin Complex

The uPAR is a GPI-anchored protein present on the surface of endothelial cells, leukocytes, and many other cell types. When the inactive pro-form of u-PA is secreted, it binds to uPAR and can subsequently be activated. A number of proteases have been reported to activate u-PA on the cell surface, including cathepsin B and kalikrein. Cathepsin B is found on various cells including endothelial cells. Cathepsin B is primarily a lysosomal enzyme, but it can bind to annexin II heteromer and p11 protein on the cell surface and activate uPAR-bound u-PA.103 The uPAR–Integrin Complex

Figure 2. The u-PA receptor acts as a coordinating protein in the assembly of a multiprotein complex involved in cell adhesion and cell signaling, and supports u-PA activation and removal of u-PA:PAI-1 complexes.

The uPAR–Integrin Complex

The uPAR acts here as an organizing center being able to form noncovalent complexes with integrins, LRP-like proteins and u-PA or urokinase plasminogen activator (uPA):PAI-1 (Figure 2). Such complexes also occur on endothelial cells.

Figure 2.

Pericellular Proteases in Angiogenesis

van Hinsbergh et al

Pericellular Activities of MMPs and MT-MMPs

It is likely that also other membrane-associated metalloproteinases, such as MT-MMPs, 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 αvβ3-integrin, which aids in focusing proteolytic activity.40,111 MT1-MMP colocalizes with β1-integrins in cell-cell contacts, whereas it was encountered with αvβ3-integrins in migrating endothelial cells.112,113 Neovascularization after myocardial infarction depends equally on u-PA/plasmin activities as on MMPs.22 However, one cannot discriminate on the basis of the data available 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.

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addition, MT1-MMP can process several membrane proteins. This includes the maturation of αv-integrins and the shedding of tissue transglutaminase, syndecan-1, and CD44. CD44 cleavage appeared to be needed for migration to proceed, because a mutant CD44H that could not be cleaved by MT1-MMP prevented cell migration. Subsequently, MT1-MMP is internalized via caveolae and clathrin-coated vesicles. The internalized MT1-MMP can either be degraded in lysosomes or be recycled to the plasma membrane. Internalization appears to be required for cell migration. Possibly the treadmilling of a complex of MT1-MMP and adjacent (receptor) proteins is required for the locomotion of the cell. Although most of these data were obtained from tumor cells and fibroblasts, it is likely that a similar mechanism also acts in endothelial cells. The important role of MT1-MMP in endothelial cell migration and angiogenesis was already mentioned. Galvez et al demonstrated that internalization of MT1-MMP via caveolae was involved in MT1-MMP-mediated migration of endothelial cells in a collagen substrate/matrix.

The mechanisms of MT1-MMP and u-PA/uPAR have much in common. Prager et al showed that the uPAR is redistributed to focal adhesions at the leading edge of endothelial cells in response to VEGF, and that subsequent cell migration depended on u-PA activation, PAI-1 interaction and internalization of the uPAR. VEGF-dependent activation of the uPAR-bound u-PA activation depended on a change in integrin affinity and MMP-2 activity bound to MT1-MMP on these cells. Other investigators reported on MT-MMPs and u-PA/plasmin as additionally acting mechanisms, each of which predominated depending on the matrix conditions. Furthermore, MT1-MMP has been implicated in the activation of MMP-2 and MMP-13 in and the processing of cellular receptors and membrane-bound proteins, as already mentioned above.

**Pericellular Activity of Cathepsin B**

In tumor cells the secreted procathepsin B binds to the cell surface via the light chain of the annexin II heterotrramer and the therewith-associated protein p11, which facilitates its conversion into active cathepsin B. This complex associates with lipid raft structures, similar as the uPAR complex. Cathepsin B can be activated from itszymogen by various proteases including t-PA, u-PA, cathepsins G and D, and elastase. Cathepsin B has also been indicated to activate uPAR-bound pro-u-PA and thus to enhance angiogenesis. Rao and colleagues have shown in a series of elegant experiments that simultaneous inhibition of cathepsin B and uPAR by siRNAs markedly reduced the growth of gliomas and their vasculature in mice in vivo, and that the simultaneous inhibition of the expression of both proteins was more effective than inhibition of one of them. Similarly, they showed that the simultaneous inhibition of cathepsin B and MMP-9 also inhibited glioma growth in mice.

Kostoulas et al proposed a potent additional mechanism by which cathepsin B may enhance angiogenesis, namely degradation of TIMP-1 and TIMP-2. This degradation leads to increased activities of MMPs. Furthermore, cathepsin B is involved in the activation of growth factors.

**Other Cysteine Cathepsins and Angiogenesis**

Other cathepsins may also be active pericellularly either after binding to membrane-bound chaperones or by association with a proton-pump, which causes a local acidification. The cysteine cathepsins B, K, L, and H are highly expressed in various tumor cells. A number of studies show the presence of cathepsins in the vasculature of human tumors (incl. glioma and prostate) associated with an increase in angiogenesis and tumor growth. In addition to the already mentioned studies on cathepsin B, the expression and role of other cathepsins in tumor angiogenesis was further investigated in RIP1-Tag2 mice. These mice develop multiple pancreatic islet tumors as a consequence of expressing the SV40 T antigen (Tag) oncogene in insulin-producing β-cells. The cathepsins B, H, L, and XZ were expressed in the tumor cells, infiltrating leukocytes, and the endothelial cells of the tumor. Inhibition of cathepsin activity by a broad-spectrum cysteine cathepsin inhibitor reduced tumor vascularization and vascular branching during pancreatic islet tumorigenesis. 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. Endothelial progenitor cells express various cysteine cathepsins, of which cathepsin L was shown to contribute to angiogenesis in mice. Mice deficient of cathepsin S show an impaired development of microvessels during wound repair. A role of cathepsin S in angiogenesis was underscored by the in vitro finding that endothelial tube formation and invasion in Matrigel and collagen 1 matrix was facilitated by cathepsin S. These data strengthen the idea that cathepsins indeed play a role in angiogenesis in tumors and tissue repair.

**Angiogenesis Enforced: Recruitment of Bone Marrow-Derived Cells**

Leukocytes and endothelial progenitor cells can contribute to the initiation and guidance of new blood vessels. u-PA/uPAR and MT1-MMP play a role in monocyte recruitment during...
inflammation, as MMP-9 does also. Monocytes produce various pro-angiogenic factors. Furthermore, a special population of CD34+ cells which can acquire endothelial-like properties, such as the expression of VE-cadherin and VEGF receptor-2 (kdrl) are thought to markedly influence the progression of angiogenesis. Their absence or dysfunction is associated with impaired vascularization in cardiac and diabetes patients. MMP-9 plays a role in the recruitment of these so-called endothelial progenitor cells (EPCs). Furthermore, these cells produce high amounts of cathepsins including the angiogenesis-stimulating cathepsin L. For a discussion of the types of circulating endothelial/progenitor cells the reader is referred to Ingram et al.

MMP-9 plays a role in the mobilization of hemopoietic and endothelial progenitor cells from the bone marrow. After suppression of the vascular niche, the transfer of hemopoietic and endothelial precursor cells to the proliferation compartment in the bone marrow requires cell activation by soluble c-kit ligand, which is set free by MMP-9. Deficiency of MMP-9 in mice retards recruitment of EPCs and angiogenesis. Similarly, MMP-9 plays a crucial role in the recruitment of bone marrow-derived leukocytes to the vasculature of neuroblastomas. This role appears critical for the formation of a mature vasculature and coverage of endothelial capillaries by pericytes. Heissig et al recently proposed an amplifying mechanism, in which: (1) circulating VEGF induced MMP-9 expression in the bone marrow; (2) MMP-9 released c-kit ligand; (3) c-kit activation recruits hemopoietic, endothelial and mast cell progenitor cells; and (4) mast cells accumulating in the angiogenic area produce large amounts of VEGF. This cycle was elevated by low-dose irradiation.

Not only MMP-9 is involved in EPC-enhanced angiogenesis. Urbich et al found that cathepsin L is required for EPC-induced neovascularization. They selected cathepsin L as a potential regulator of EPC-enhanced neovascularization after studying the expression profiles of various proteases and protease inhibitors in EPCs, endothelial cells and CD14+ monocytes. These EPCs are able to stimulate neovascularization and blood flow in the ischemic murine hindleg after their injection in affected hindleg. A high expression of a number of cysteine cathepsins occurred specifically in EPCs, including cathepsins D, H, L, K, and X/Z, as became obvious from mRNA array analysis. The antigen and activity of cathepsin L was considerably higher in EPCs than in monocytes and endothelial cells. This activity may be, at least in part, extra- or pericellular. Mature cathepsin L can remain active extracellularly at neutral pH by the chaperone action of a p41 splice variant of the MHC class II-associated invariant chain, which indeed is strongly expressed in EPCs. Such activity may facilitate EPC invasion, remodeling of matrix collagens and gelatin, and neovascularization. Deficiency of cathepsin L in mice caused a significant impairment of blood flow restoration in ischemic limbs, indicative for an impaired neovascularization. Furthermore, neovascularization was reduced in mice treated with bone marrow derived cells deficient of cathepsin L as compared with wild-type cells. Cathepsin L was also required for the recruitment and infiltration of EPCs into glioma xenografts and their vasculature in mice. The target via which cathepsin L stimulates angiogenesis is not yet resolved.

### Switching Off Angiogenesis: Matrikines as Endogenous Inhibitors Generated by Proteases

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 (Table). A thrombospondin fragment was initially recognized in 1990. In a search for primary tumor factors that suppressed the growth of metastases, Folkman et al isolated angiostatin and endostatin from the urine and tumor tissue of mice. When these compounds were administrated to tumor-bearing mice, these investigators observed growth arrest, regression, and dormancy of the tumor grafts.

Presently, a considerable number of extracellular matrix fragments are known that inhibit angiogenesis (Table). Several of them act as competitive inhibitors of specific integrin-matrix interactions, whereas others affect endothelial metabolism. Endostatin, the C-terminal part of collagen type XVIII, is generated by cathepsin-L and elastase. It inhibits VEGF-induced endothelial migration and induces apoptosis. An important aspect of the biological activity of endostatin is its ability to bind via heparin sulfates to α5β1-integrin. This complex affects cellular signaling through caveolin and Src and disturbs the...
proper organization of the F-actin cytoskeleton.\textsuperscript{153} The collagen type IV fragment tumstatin inhibits focal adhesion kinase (FAK) through interaction with\(\alpha\beta3\)-integrin interaction. It inhibits activation of various signaling kinases (PI3-kinase, Akt, mTOR), and causes endothelium-specific inhibition of protein synthesis.\textsuperscript{154} It is likely that additional extracellular matrix fragments will be recognized that act as angiogenesis modulators.

Proteolytic fragments of factors involved in the hemostatic system represent an additional group of anti-angiogenic regulatory molecules (Table).\textsuperscript{155} Anti-angiogenic activities in vitro as well in vivo have also been identified in the proteolytically generated kringle domains of plasminogen.\textsuperscript{147,156} Prothrombin,\textsuperscript{157} HMW-kininogen,\textsuperscript{158} and u-PA.\textsuperscript{159} Although angiostatin (kringles 1 to 4, generated from plasminogen by various proteases, including MMP-3, MMP-7, MMP-9 and u-PA) was the first degradation product recognized to have anti-angiogenic properties, the kringle-5 domain of human plasminogen has been shown to possess powerful anti-angiogenic activity of the plasminogen fragments as well.\textsuperscript{156,160} These plasminogen-derived kringles target to several proteins on the endothelial membrane including ATP synthase, angiopotin,\(\alpha\beta3\)-integrin, and the HGF receptor c-met and induce endothelial cell death.\textsuperscript{160} The N-terminal 24 amino acid peptide of the \(\alpha\)-chain of human fibrinogen has been recognized to harbor the anti-angiogenic activity that was originally observed to be present in fibrinogen degradation product E.\textsuperscript{161}

### Stabilization of New Vessels: Proteases and the Recruitment of Pericytes

The present paradigm of angiogenesis considers the smooth-muscle-like pericyte as key regulator of the stabilization of newly formed vessels.\textsuperscript{162} MMP-9 was present in pericytes present in the stroma of tumors of breast cancer patients.\textsuperscript{163} The recruitment of pericytes was disturbed in animals that were made deficient of MMP-9.\textsuperscript{164} This markedly affects the stability of vessels and the degree of vascularization of neuroblastomas.\textsuperscript{164} Aminopeptidase A was also present in activated pericytes in newly formed vessels and the degree of vascularization of neuroblastomas.\textsuperscript{164} Aminopeptidase A was also present in activated pericytes in various pathological conditions associated with angiogenesis, whereas its expression was low in pericytes of quiescent vessels.\textsuperscript{165} The mechanism how this protease contributes to angiogenesis has not yet been investigated. 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.\textsuperscript{166,167}

Without pericyte covering, the immature vessels remain dependent on the continuous exposure to angiogenic growth factors.\textsuperscript{162} Once their supply ceases, the endothelial cells do no longer survive.\textsuperscript{168,169} Caspases are involved in endothelial apoptosis, while other proteases take part in the degradation and removal of the cell remains.

### Increasing Proximal Blood Supply

When angiogenesis is effective many new microvascular structures are formed. However, to acquire an adequate perfusion of these newly formed vessels, the proximal vascular tree has to be remodeled. Enlargement of small arteries and arterioles will be necessary to provide a suitable amount of blood per unit of time. This requires remodeling of the smooth muscle cell layer of these vessels, a process that also involves various proteases that were described above to be involved in matrix remodeling and migration. The reader is referred to the work of Schaper et al\textsuperscript{170,171} for further details.

### Perspective

From this article, it is clear that proteases can influence neovascularization in many different ways, both in development and particularly in pathological conditions. Because they are major regulators of tissue destruction and cell migration, it was initially expected that inhibition of matrix-degrading proteinases would be beneficial to counteract growth of tumors and their vasculature. Although promising results were obtained in animal studies, clinical trials failed to show an improving effect and often resulted in unacceptable side effects.\textsuperscript{172} This has stimulated further research, from which new families of proteases, such as MT-MMPs, ADAM, and ADAMTS, were discovered. More importantly, a new view has become apparent on how proteases modulate growth factors and cytokines and generate new biologically active fragments from matrix and circulating proteins. Protease inhibitors also appear to have additional unexpected effects by themselves, of which much has still to be learned. The new insights in the modulation of cytokines by proteases contribute to the understanding of inadequate cytokine-mediated response in disease, as well as in improving the use of growth factors and cytokines in treating disease. In addition, the new biological entities that inhibit or stimulate angiogenesis can provide new leads for developing agents that inhibit or stimulate angiogenesis, depending on the disease to be combated.

The complexity of the effects of proteases in disease is enhanced by parallel activities of proteases from similar or even different families. Better understanding of these mechanisms will be helpful in establishing strategies to deal with these redundancies and in avoiding side effects. The use of agents that target to specific cells and receptors on the cell surface\textsuperscript{173} and combined use of either specific protease inhibitors or virally and nonviral delivered siRNAs\textsuperscript{127} are options that may contribute to reduce the burden of specific malignancies.

Although mainly studied from the angle of tumor growth and treatment, the role of proteases in angiogenesis extends to many other aspects of human life, starting with implantation of the embryo and placentation and growth of the fetus. Proper wound healing and tissue repair after inflammation depend on proteases. Furthermore, the lack of blood perfusion or the excessive growth of vessels is pivotal in many pathological conditions.\textsuperscript{174,175} It is likely that the substrates and products of proteases will be the preferred targets for treating these diseases, but successful applications often depend on knowledge how these proteins and peptides are activated, altered, and generated by proteases.

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