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Matrix metalloproteinases and angiogenesis

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Abstract

Matrix metalloproteinases (MMPs) are a family of enzymes that proteolytically degrade various components of the extracellular matrix (ECM). Angiogenesis is the process of forming new blood vessels from existing ones and requires degradation of the vascular basement membrane and remodeling of the ECM in order to allow endothelial cells to migrate and invade into the surrounding tissue. MMPs participate in this remodeling of basement membranes and ECM. However, it has become clear that MMPs contribute more to angiogenesis than just degrading ECM components. Specific MMPs have been shown to enhance angiogenesis by helping to detach pericytes from vessels undergoing angiogenesis, by releasing ECM-bound angiogenic growth factors, by exposing cryptic proangiogenic integrin binding sites in the ECM, by generating promigratory ECM component fragments, and by cleaving endothelial cell-cell adhesions. MMPs can also contribute negatively to angiogenesis through the generation of endogenous angiogenesis inhibitors by proteolytic cleavage of certain collagen chains and plasminogen and by modulating cell receptor signaling by cleaving off their ligand-binding domains. A number of inhibitors of MMPs that show antiangiogenic activity are already in early stages of clinical trials, primarily to treat cancer and cancer-associated angiogenesis. However, because of the multiple effects of MMPs on angiogenesis, careful testing of these MMP inhibitors is necessary to show that these compounds do not actually enhance angiogenesis.

Keywords: matrix metalloproteinases (MMPs) • angiogenesis • tumor angiogenesis • MMP inhibitors • extracellular matrix remodeling • tissue inhibitors of metalloproteinases (TIMPs) • thrombospondins

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Introduction

Angiogenesis is important for vascular remodeling in the embryo as well as in female reproductive cycles and wound healing in the adult. However, in certain pathological conditions, such as rheumatoid arthritis, diabetic retinopathy, psoriasis, hemangiomas, and cancer, aberrant angiogenesis occurs [1]. An understanding of normal and abnormal angiogenesis is required in order to develop therapeutic strategies to combat these pathologies. Since angiogenesis involves migration/invasion of endothelial cells into surrounding stroma/tissues, proteases such as the matrix metalloproteinases (MMPs) are critically important. However, recently it has become clear that MMPs' role(s) in angiogenesis is more complex than simply degrading the extracellular matrix (ECM) to facilitate invading endothelial cells. Various MMPs are also necessary for releasing ECM-sequestered proangiogenic factors, processing of growth factors and receptors, including integrins and adhesion receptors, and for generating endogenous antiangiogenic compounds [1–3]. This review will summarize the various roles of MMPs in facilitating angiogenesis as well as inhibiting angiogenesis.

Process of angiogenesis

Vasculogenesis is the formation of new blood vessels *de novo* by differentiation of progenitor angioblasts to form the primary capillary plexus in the developing embryo. After formation of the early vascular plexus, endothelial cells proliferate and new capillaries are formed by sprouting or non-sprouting mechanisms from an existing blood vessel, which is the process of angiogenesis. Non-sprouting angiogenesis occurs by the formation of transcapillary posts of ECM and the proliferation of endothelial cells within an existing vessel, thereby splitting the vessel into two or more capillaries. This type of angiogenesis predominates in the lung during organogenesis [4]. Sprouting angiogenesis (illustrated in Fig. 1) involves the proteolytic degradation of the basement membrane surrounding endothelial cells in a vessel, followed by migration and proliferation of endothelial cells into the adjacent stroma. Differentiation and maturation of the endothelial cells, lumen formation, recruitment of pericytes, and coalescence of tubes into loops completes the process of new blood vessel formation. Sprouting angiogenesis occurs in the yolk sac and in the embryo during later organogenesis, especially in the brain [4]. In the adult mammal, the vasculature is normally quiescent, except during highly regulated processes of female reproductive cycles and wound repair, and in pathological situations such as ophthalmic and rheumatic diseases and tumor angiogenesis [4–6].

The signals that initiate and sustain angiogenesis are multiple and complex. Proangiogenic cytokines and growth factors include vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), angiopoietins, transforming growth factor- (TGF β), platelet-derived growth factors (PDGFs), tumor necrosis factor-α (TNF α), epidermal growth factor (EGF), interleukin-8 (IL-8), and angiogenin, which are secreted by inflammatory cells (*e.g.*, mast cells and macrophages), pericytes, keratinocytes (during epidermal wound healing), or tumor cells. Some of these factors act directly by binding to their respective receptors on endothelial cells to induce proliferation and/or migration, while others act on local stromal or inflammatory cells to stimulate angiogenesis [5, 7]. ECM and basement membrane components also transduce both proangiogenic and antiangiogenic signals by binding to integrins on endothelial cells. For example, intact fibrillar type IV collagen is bound by integrins α1β1 and α2β1 expressed on angiogenic endothelial cells and induces their proliferation and migration, while degraded type IV collagen does not bind these integrins and instead binds to αvβ3 integrin, inhibiting endothelial cell migration and proliferation [8]. The ECM in addition acts as a sequestration/storage compartment for angiogenic growth factors such as VEGF, basic FGF (bFGF), and TGF 1, which can be released by proteolytic degradation of the ECM [8].

There are also multiple endogenous inhibitors of angiogenesis. Bioactive cleaved forms of collagens, which include endostatin, tumstatin, arrestin, and canstatin, bind to endothelial cell surface integrins to inhibit proliferation and migration [8, 9]. Angiostatin, a proteolytic internal fragment of plasminogen, binds to ATP synthase and to angiomotin on the surface of endothelial cells to inhibit cell proliferation and migration, respectively [10, 11]. Other angiogenesis inhibitors are compounds that

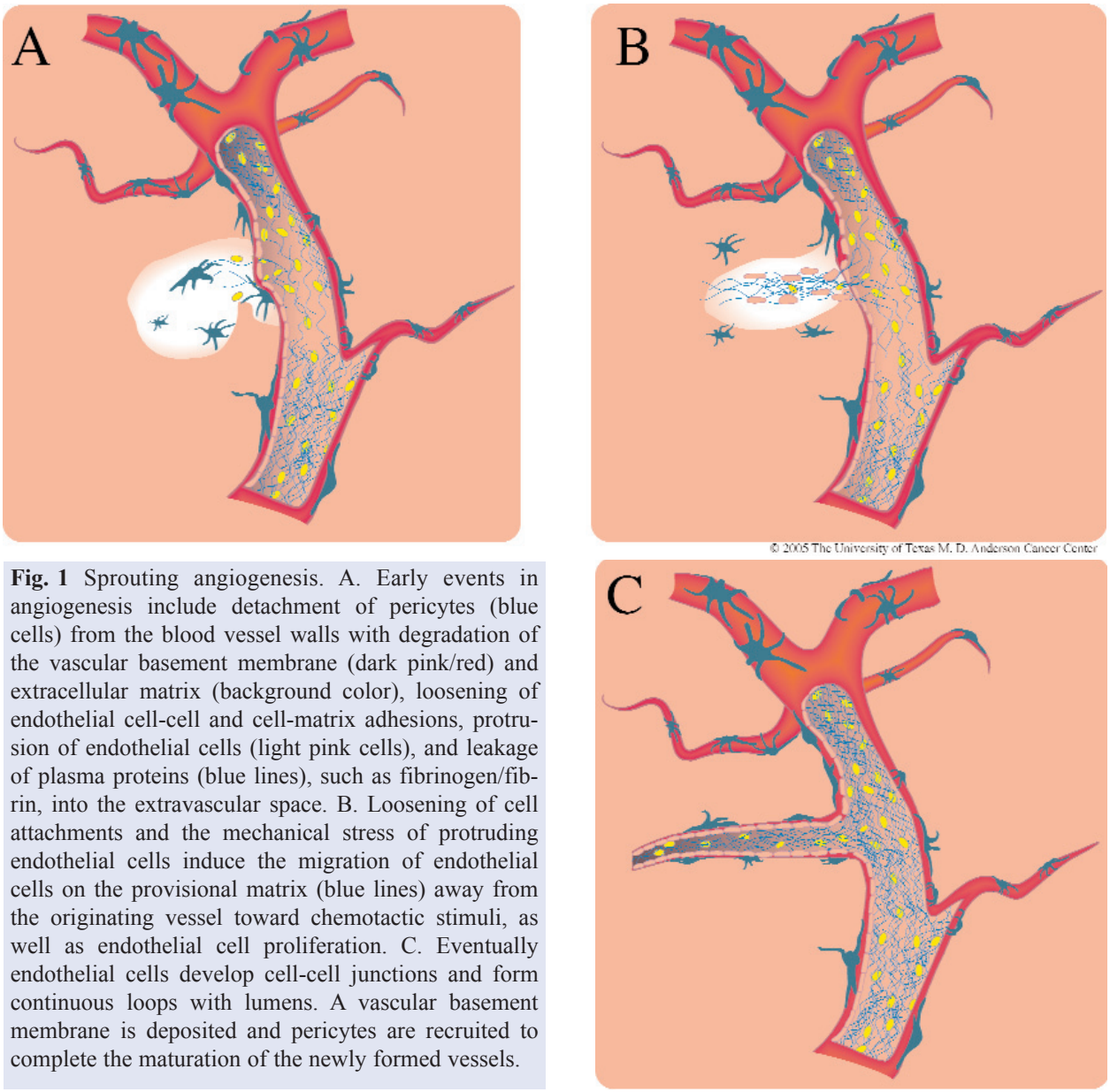

Fig. 1 Sprouting angiogenesis. A. Early events in angiogenesis include detachment of pericytes (blue cells) from the blood vessel walls with degradation of the vascular basement membrane (dark pink/red) and extracellular matrix (background color), loosening of endothelial cell-cell and cell-matrix adhesions, protrusion of endothelial cells (light pink cells), and leakage of plasma proteins (blue lines), such as fibrinogen/fibrin, into the extravascular space. B. Loosening of cell attachments and the mechanical stress of protruding endothelial cells induce the migration of endothelial cells on the provisional matrix (blue lines) away from the originating vessel toward chemotactic stimuli, as well as endothelial cell proliferation. C. Eventually endothelial cells develop cell-cell junctions and form continuous loops with lumens. A vascular basement membrane is deposited and pericytes are recruited to complete the maturation of the newly formed vessels.

disrupt growth factor signaling, such as soluble forms of VEGF receptors, VEGFR-1 (Flt-1) and neuropilin-1 [12]. Both angiopoietin-1 (Ang1) and Ang2 bind to the angiopoietin receptor, Tie2, which is expressed on endothelial cells, but only Ang1 can activate Tie2 signaling [5]. Thus, Ang2 is a natural antagonist of Ang1 and transgenic overexpression of Ang2 disrupts developmental angiogenesis [13]. Proteins with thrombospondin-type motifs, such as thrombospondin-1 and -2 (TSP-1 and -2), platelet factor-4, and several members of the metallospondin/ADAMTS (A Disintegrin And Metalloprotease with ThromboSpondin-like repeats) family also show antiangiogenic activity [14]. Thrombospondin-1 binds to CD36, a class B scavenger receptor and collagen-binding protein, on endothelial cells, which results in inhibition of cell migration and induction of apoptosis [14].

Physiological angiogenesis (wound angiogenesis)

Thus, the process of angiogenesis is controlled by the balance and interaction of signals from pro- and

antiangiogenic factors from and to different cell types. As an example, angiogenesis after wounding or injury is initiated by the disruption of epidermal (*e.g.* in skin wounding) and vascular basement membranes, which releases ECM-bound angiogenic growth factors such as the FGFs [15]. Simultaneously, damage to blood vessels leads to coagulation where platelets adhere, aggregate, and degranulate, releasing numerous angiogenic mediators, including TGF β, PDGF, and VEGF, as well as cytokines that recruit macrophages and monocytes in an inflammatory response [5]. VEGF acts on endothelial cells to induce cell migration and proliferation, as well as acting as a vascular permeability factor, which allows leakage of plasma proteins such as fibrinogen/fibrin that create a temporary support structure for migrating epithelial cells, leukocytes, and endothelial cells [5, 16].

Within the granulation tissue, activated macrophages release bFGF and TNF α, as well as nitric oxide (NO) [17]. NO induces vasodilation and VEGF transcription by endothelial cells [16]. In a positive feedback loop, VEGF induces expression of endothelial NO synthase (eNOS) resulting in additional production of NO [16]. Hypoxia within the granulation tissue induces VEGF transcription and secretion by macrophages and reepithelializing keratinocytes *via* the transcription factor hypoxia-inducible factor-1α (HIF-1 α) [17, 18]. Also TNF α, as well as TGF 1, induces VEGF expression by keratinocytes [19]. TNF α and TGF β further contribute to angiogenesis by inducing expression in dermal fibroblasts and keratinocytes of MMP-9 capable of degrading basement membrane and ECM components [20].

While VEGF is responsible for increasing vascular permeability, Ang1 through its receptor Tie2 inhibits vascular leakage, but also is chemotactic to endothelial cells and induces endothelial cell sprouting and secretion of proteases plasmin and MMP-2, stimulating angiogenesis [21–23]. Ang1 is constitutively expressed in the adult, while Ang2 is highly expressed at angiogenic and vascular remodeling sites and antagonizes Ang1 actions on Tie2 [13]. However, Ang2 contributes to angiogenesis by promoting detachment of smooth muscle cells and loosening of the underlying matrix [16]. VEGF upregulates the expression of specific integrins (α1β1, α2β1, and αvβ3) on microvascular endothelial cells, which allows the cells to adhere and migrate across the provisional wound matrix containing native type I collagen, fibronectin, fibrin, and osteopontin [24]. Integrin αvβ3, which binds Arg-Gly-Asp (RGD)-containing proteins including vitronectin, fibronectin, fibrin, osteopontin, and denatured collagens, is specifically expressed on the tips of sprouting endothelial cells and on newly formed blood vessels in the granulation tissue, while its expression is lost in mature, quiescent blood vessels [5, 25]. Other angiogenic growth factors, bFGF, TNF α, and IL-8, induce expression of integrin α5β1, another fibronectin receptor, on endothelial cells [26]. Crosstalk between integrin signaling pathways also influence angiogenesis. Endothelial cell migration and angiogenesis mediated by integrin αvβ3 is potentiated by fibronectin ligation to integrin α5β1 [27].

Finally, migrating endothelial cells assemble into solid cords, forming junctions with adjacent endothelial cells *via* vascular endothelial-cadherin (VE-cadherin) and connexins, and then a lumen is formed, which is regulated by VEGF, Ang1, and integrins αvβ3 and α5β1 [16]. Mechanical/shear stress of extruding endothelial cells induces expression and secretion of PDGF-B, which recruits and induces proliferation and differentiation of pericytes and smooth muscle cells expressing the PDGF receptor β, thereby completing maturation of the new blood vessels [4, 16, 25]. Vascular smooth muscle cells inhibit endothelial cell proliferation and migration, thereby stabilizing the new vessels [16]. Interactions between endothelial cells and pericytes/smooth muscle cells further induces and activates TGF β, which enhances ECM deposition and contributes to vessel maturation and stabilization [16, 25, 28].

Tumor angiogenesis

In pathological angiogenesis, such as psoriasis, ophthalmic and rheumatic diseases, inflammation is often involved, and the release of proangiogenic factors and proteases by the infiltrating inflammatory cells provide the signals for aberrant angiogenesis [4, 15, 29]. On the other hand, tumor angiogenesis involves an "angiogenic switch" that shifts the balance to more pro- than antiangiogenic signals

and often occurs at an early, premalignant stage [6, 29, 30]. Signals that trigger the angiogenic switch in tumors include metabolic stress, such as hypoxia and acidosis, genetic mutations that activate certain oncogenes or inactivate/delete tumor suppressor genes, and the presence of an immune/inflammatory response within the tumor/lesion [29].

Without adequate vascular perfusion, the high proliferation rate in hyperplastic/dysplatic lesions is balanced by increased differentiation, apoptosis, and/or necrosis, and so tumor volume is limited. Developing hypoxia in a growing tumor mass up-regulates VEGF expression by tumor cells and when VEGF levels become high enough to overcome endogenous antiangiogenic signals, angiogenesis is initiated [6, 30]. Hypoxia-activated HIF-1α also induces transcription of other angiogenic genes including NOS, PDGF-B, and Ang-2 [29].

Many tumors have been found to express various pro-angiogenic growth factors, such as FGFs and VEGF [30]. Transformation of nontumorigenic epithelial cells and fibroblasts with mutant ras oncogenes has been shown to up-regulate VEGF expression, which correlates with acquisition of a tumorigenic and angiogenic phenotype [31, 32]. Loss of the tumor suppressor gene, p53, which frequently occurs in human cancers, can also contribute to the angiogenic switch by enhancing HIF-1α levels and thereby induction of VEGF expression, as well as by down-regulating the expression of the angiogenesis inhibitor, TSP-1 [30, 33]. TSP-1 expression has been found to be inversely correlated with malignant progression in melanoma, breast, and lung cancer cell lines [30]. In early androgen-sensitive prostate cancer, TSP-1 expression was shown to be inversely correlated with vessel density, and androgen deprivation resulted in increased TSP-1 expression and vascular regression [34]. Expression of VEGF by tumor cells also induces vascular permeability, which leads to extravasation of plasma proteins providing a provisional matrix for migrating endothelial cells [30].

In addition to inducing angiogenesis from existing blood vessels, angiogenic factors released from tumor cells can recruit bone marrow-derived circulating endothelial progenitor cells, which express VEGFR-2 (Flk-1), as well as VEGFR-1-expressing hematopoietic stem and progenitor cells that facilitate the incorporation of endothelial progenitor cells into tumor vessels [35]. Another mechanism tumor cells use to enhance perfusion in solid tumors such as melanoma is the capacity of certain cancer cells to mimic endothelial cells to form ECM-rich, fluid-conducting networks, called vasculogenic mimicry [36]. Tumor blood vessels are different than normal blood vessels in that the tumor vasculature is chaotic with tortuous, leaky, and dilated or uneven in diameter vessels with reduced attachment of pericytes or pericyte function in part due to dysregulated expression of VEGF and other angiogenic factors by tumor cells [29, 37].

MMPs, regulation of activity

MMPs, also called matrixins, are a family of over 20 zinc-containing endopeptidases that are capable of degrading various components of the ECM [38]. All are produced as latent, pro-enzymes, which must be proteolytically processed to be activated. MMPs have been subdivided into at least five groups based on their structure and/or substrate specificities (Fig. 2). The simplest structural subclass of MMPs is the matrilysins, which consist of a signal peptide, propeptide domain, and catalytic domain with the zinc-binding site [39, 40]. The collagenases, which in addition to the minimal domain structure also contain a simple hemopexin-like domain connected to the catalytic domain via a proline-rich hinge region, degrade the native helix of types I, II, III, and other fibrillar collagens [39, 41]. The stromelysins have similar structural domains as the collagenases, but like the matrilysins, have a broad substrate specificity and degrade many ECM proteins, including proteoglycans, fibronectin, and laminin [2, 39]. The gelatinases contain an additional region of three fibronectin type II repeats within their catalytic domains and show a preference for denatured collagens (gelatin) and also degrade types IV, V, VII, and X native collagens, fibronectin, and laminin [39–41]. The fifth major subgroup of MMPs is the membrane-type MMPs (MT-MMPs). These MMPs are bound to the cell surface *via* a C-terminal transmembrane domain or glycosylphosphatidylinositol anchor [41, 42] and degrade gelatin, fibronectin, and aggrecan as well as other ECM substrates [39, 40]. Other MMPs not shown in Fig. 2 include metalloelastase (MMP-12) and enamelysin (MMP-20), which have specialized

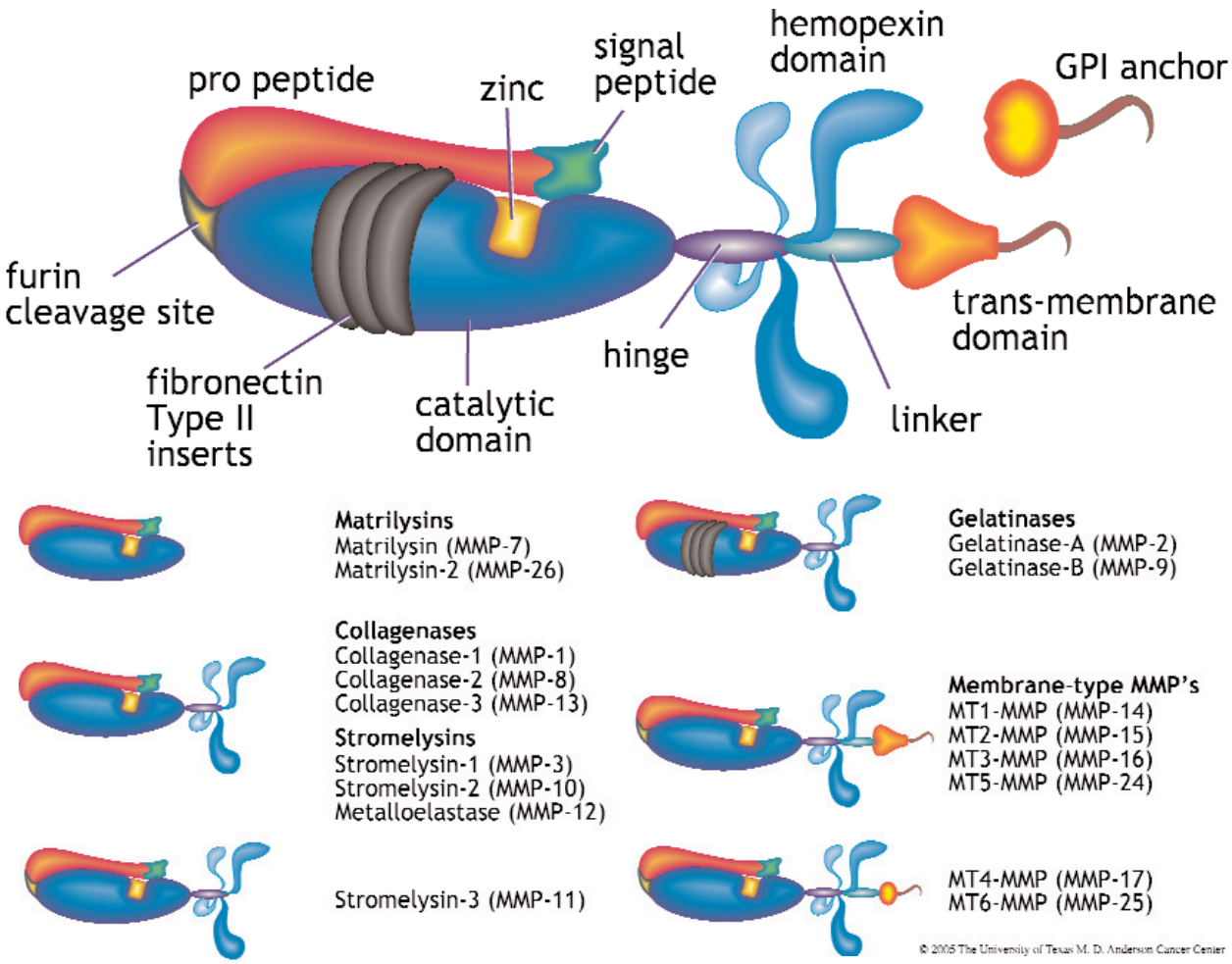

Fig. 1 Basic structural domains of the MMPs. The matrilysins contain the minimal domain structure consisting of a signal peptide, a propeptide domain, and a catalytic domain with a highly conserved zinc-binding site. A conserved cysteine in the propeptide domain coordinates with the zinc in the active site to maintain latency of the proMMPs. The collagenases and stromelysins contain in addition to the minimal domain structure a hemopexin-like (PEX) domain in a four-bladed propeller-type structure connected to the catalytic domain *via* a hinge region. The gelatinases have three fibronectin type II repeats within their catalytic domains, which allows binding to denatured collagens (gelatin). The MT-MMPs are tethered to the cell surface *via* a transmembrane domain with a short cytoplasmic tail or *via* a glycosylphosphatidylinositol (GPI) membrane anchor. Some of the MMPs contain a furin-cleavage site between their propeptide and catalytic domains allowing activation by furin-type convertases.

functions in macrophage-mediated proteolysis/migration and in tooth enamel formation, respectively [43, 44], and MMP-19, MMP-23, and epilysin (MMP-28) [39].

The pro-domain of all MMPs contains a conserved cysteine residue, called the "cysteine switch", whose sulfhydryl group coordinates with the zinc ion in the catalytic site to maintain latency [38]. Disruption of this cysteine-zinc binding by physical or chemical means is the first step in activation of the MMPs [2]. The hemopexin (PEX) domain, which forms a four-bladed propeller structure made of β-sheets, along with an appropriate hinge region allows collagenolytic MMPs to unwind/distort the triple helix of fibrillar collagens so the catalytic domain can cleave them [45]. The PEX domain is also necessary for MMP binding to a number of other proteins, including integrins, cell surface receptors, and tissue inhibitors [2, 46–49]. On the other hand, the fibronectin-like domain of the gelatinases is important for binding to gelatin [45]. All of the MT-MMPs, stromelysin-3 (MMP-

11), MMP-23, and epilysin (MMP-28) also have a furin recognition sequence between their propeptide and catalytic domains, allowing cleavage/activation by furin convertase enzymes in the Golgi apparatus [41, 42].

In addition to degrading ECM components and activating other MMPs, MMP activity is responsible for making available active growth factors and cytokines. MMP degradation of insulin-like growth factor (IGF) binding proteins releases active IGFs, degradation of the proteoglycan perlecan in vascular basement membranes releases FGFs, and degradation of latent TGFβ binding proteins, such as decorin, releases latent TGF β, while MMP-2 and MMP-9 proteolytically activate latent TGF β1 and TGF β2 [2, 39]. MMP-3 and MMP-7 have been shown to cleave the membrane-bound precursor of heparin-binding EGF (HB-EGF), releasing active HB-EGF, while TNF α is released from the cell surface by MMP-1, -3, and -7 [2]. Relevant to angiogenesis, MMP-9 cleaves the pro-inflammatory, pro-angiogenic cytokine IL-8, which increases its activity tenfold, as well as degrading and inactivating the angiogenesis inhibitor platelet factor-4 [50]. On the other hand, MMP-2 cleaves the FGF receptor 1 (FGFR1), releasing the soluble ectodomain of FGFR1 that can still bind FGFs, but lacks signaling capacity [51].

Except for the MMPs activated intracellularly by furin proteases, the other MMPs are secreted as inactive zymogens and must be activated in the extracellular space by proteolytic cleavage of the N-terminal propeptide domain [38, 42]. Plasmin, generated from plasminogen through the action of urokinase-type plasminogen activator (uPA), can initiate an MMP activation cascade, activating proMMP-1 and proMMP-3 [41, 52, 53]. Activated MMP-3 can in turn activate proMMP-1 and proMMP-9 [52, 53]. MT1-MMP activates proMMP-2 as well as proMMP-13 at the cell surface [54, 55]. Activated MMP-2 and MMP-13 can both in turn activate proMMP-9 [56, 57].

MMP activity is additionally regulated by endogenous inhibitors, primarily the tissue inhibitors of metalloproteinases (TIMPs). There are 4 TIMPs, TIMP-1 to -4, which each consist of an N-terminal domain responsible for their MMP inhibitory activity and a C-terminal domain [58]. Although all of the TIMPs bind tightly to most MMPs, they have differential inhibitory activity against different MMPs. For example, TIMP-2 and TIMP-3, but not TIMP-1, are efficient inhibitors of the MT-MMPs [58]. TIMP-2 has a specialized role in the activation of proMMP-2 by MT1-MMP. The N-terminal domain of TIMP-2 forms an inhibitory complex with the active site of MT1-MMP, while the C-terminal domain interacts with the PEX domain of MMP-2. A second TIMP-2-free MT1-MMP molecule is recruited to the complex and cleaves off the pro-domain of MMP-2 [58]. TIMPs form other non-inhibitory complexes *via* their C-terminal domains: TIMP-1 forms a complex with the PEX domain of MMP-9, while TIMP-3 complexes with both MMP-2 and MMP-9 and binds tightly to the ECM [41, 58]. TIMPs also have other biological activities that are independent of their MMP inhibitory activity. With regard to angiogenesis, TIMP-2, but not TIMP-1, inhibits bFGF-induced endothelial cell proliferation [59]. In vascular smooth muscle cells, overexpression of TIMP-2 inhibits proliferation, while TIMP-3 induces apoptosis [60].

While the TIMPs are the primary tissue inhibitors of MMPs, in the plasma, the general protease inhibitor, α2-macroglobulin, is the predominant MMP inhibitor [46, 61]. The angiogenesis inhibitor TSP-1 has been shown to inhibit the activation of proMMP-2 and proMMP-9, while TSP-2 complexes with MMP-2 to enhance clearance by scavenger receptor-mediated endocytosis [61]. Another protein with MMP inhibitory activity is RECK (REversion-inducing Cysteine-rich protein with Kazal motifs), a glycosylphosphatidylinositol membrane-anchored glycoprotein widely expressed in human tissues [46]. RECK knockout mice die *in utero* at E10.5 and have a defect in blood vessel maturation, while overexpression of RECK in subcutaneously-injected HT1080 human fibrosarcoma cells resulted in impairment of the sprouting stage of angiogenesis [46].

MMPs are also regulated at the transcriptional and post-transcriptional levels. Expression of most of the MMPs is low in normal tissues and is strongly up-regulated when ECM remodeling is required [62]. Expression can be induced by cytokines, growth factors, chemical agents (such as tumor promoters), physical stress, activated oncogenes, and interactions with the ECM [38, 62]. Promoter regions of inducible MMPs (MMP-1, -3, -7, -9, -10, -12, and -13) contain multiple cis-acting elements

including AP-1, PEA3, Sp1, and NF-κB binding sites [62]. Stabilization of MMP-1 and MMP-3 mRNAs has been demonstrated following activation of p38α mitogen-activated protein kinase [63]. MMP-9 levels have been shown to be regulated at the level of translation efficiency [64], while translational repression can regulate protein levels of human MMP-13 [65]. Post-translational modifications of MMP-9 include glycosylation [66] and covalent linkage to neutrophil gelatinase-B-associated lipocalin in neutrophils [50] or to chondroitin sulfate proteoglycans in macrophages [67]. In endothelial cells and inflammatory cells, MMPs are stored intracellularly in secretory vesicles and so can be rapidly released upon stimulation [41, 50, 68].

MMP clearance and catabolism is mediated by the low density lipoprotein receptor-related protein, which is also responsible for clearance of TSP-2-bound MMP-2 and α2-macroglobulin-MMP complexes [46, 61, 69–71].

Proangiogenic roles of MMPs

MMPs are absolutely necessary for angiogenesis, but because of overlapping activities, most MMP knockout mice have no overt angiogenic phenotype. Only MT1-MMP knockout mice show severe defects in angiogenesis, as well as craniofacial, axial, and appendicular skeletal defects [72]. On the other hand, MMP-9 knockout mice exhibit abnormal skeletal growth plate angiogenesis and delayed ossification, which is eventually compensated to produce a normal appearing skeleton [73]. While degrading ECM components to open up an avenue for migrating endothelial cells is an essential requirement for MMPs in angiogenesis, MMPs contribute in many ways to both pro- and antiangiogenic processes. Some of these MMP-regulated processes are illustrated in Fig. 3. In normal, physiological angiogenesis there is a tightly-controlled balance between angiogenic factor signaling, MMP activity/signaling, endogenous angiogenesis inhibitors and MMP inhibitors. In pathological angiogenesis, the balance becomes perturbed.

MMPs have been shown to have multiple effects on endothelial cells themselves. MMPs are necessary for endothelial cell migration and tube formation [74]. MMPs, but not the plasminogen activator/plasmin system, are involved in endothelial cell migration and invasion of fibrin barriers, with MT1-MMP showing the greatest fibrinolytic activity [75]. MMP-7 (matrilysin) enhances endothelial cell proliferation, up-regulates endothelial expression of MMP-1 and MMP-2, and induces angiogenesis *in vivo* [76, 77]. Exogenous MMP-9 has been shown to enhance endothelial cell growth *in vitro* [78]. MMPs cleave the ectodomain of VE-cadherin, thereby breaking cell-cell adhesions [79]. MT1-MMP processes the αv integrin into two disulfide-linked fragments that retain RGD-ligand binding and this processing enhances integrin signaling through focal adhesion kinase, contributing to enhanced adhesion and cell migration on vitronectin [80]. Proteolytic cleavage of type IV collagen by MMPs exposes cryptic αvβ3 binding sites, which promotes angiogenesis [81]. This cleavage is associated with a loss of binding to integrin α1β1 and an increase in αvβ3 binding and correlates with increased MMP-2 expression and activation [81]. Exposure of these cryptic sites are found within the endothelial basement membranes in angiogenic and tumor blood vessels, but not in quiescent vessels [81]. MMP-2 binding *via* its PEX domain to αvβ3 on endothelial cells is required for angiogenesis [82]. Soluble PEX domain inhibits MMP-2 binding to αvβ3 and blocks angiogenesis [83].

MMPs involved in angiogenesis can originate from infiltrating inflammatory cells, from tumor cells, or from endothelial cells themselves. Angiogenic factors can induce the expression of MMPs in endothelial cells. Membrane vesicles containing MMP-2, MMP-9, and MT1-MMP can be found in endothelial cells, in some cases localized to invading pseudopodia, and angiogenic stimulation of cells with bFGF or VEGF results in shedding of vesicles [68, 84]. bFGF induces endothelial MMP-9 expression and promoter activity via AP-1 [85]. bFGF stimulation of endothelial cells also up-regulates expression of uPA and integrin αvβ3, which are involved in the activation cascade of MMPs and in the cell surface localization of MMPs, respectively [86]. VEGF induces expression of MMP-1 in endothelial cells [87]. While latent MMP-2 is constitutively secreted by endothelial cells, exposure of cells to type I collagen (such as after disruption of the basement membrane) or to the inflammatory cytokine TNF α up-regulates expression of MT1-MMP, which leads to activation

of MMP-2 [74]. Others have shown that three-dimensional culture of endothelial cells in type I collagen induced expression and activation of MMP-2, MT1-MMP, and MMP-13, as well as decreased expression of TIMP-2 [88, 89]. Similarly, when endothelial cells are exposed to thrombin (such as after injury and clotting), activation of MMP-2 is induced. In this case, thrombin interacts with thrombomodulin, constitutively expressed on endothelial cells, which converts protein C to the anticoagulant serine protease, activated protein C, that then activates proMMP-2 directly [74]. Three-dimensional culture of endothelial cells in fibrin gel (as encountered in granulation tissue) was shown to induce expression of MT1-, MT2-, MT3-MMP and MMP-2 and bFGF/VEGF stimulation of tube formation was dependent of TIMP-2-sensitive MMPs (*i.e.* MT1-MMP) [90]. Release of NO by inflammatory cells leads to transcriptional up-regulation of MMP-13 and its activation by endothelial cells [91]. Ang1, which induces endothelial cell sprouting, induces secretion of plasmin and MMP-2, and decreases secretion of TIMP-2 by endothelial cells [22]. Stimulation of endothelial cell migration by wound healing or by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, resulted in up-regulation of cell-surface expression and activity of MT1-MMP, which was localized to the invading lamellipodia [92].

MMPs can also originate from stromal cells. TNF α induces MMP-9 transcription (*via* NF-κB and AP-1 sites) and secretion by vascular smooth muscle cells [93], while it induces MMP-1 and MMP-3 expression in fibroblasts *via* stabilization of mRNAs [63]. In the chick chorioallantoic membrane *in vivo* angiogenesis assay, onset of angiogenesis coincided with up-regulated expression of MMP-13, which was expressed by cells of monocyte/macrophage lineages [94]. Addition of exogenous MMP-13 to the chorioallantoic membrane assay induced an angiogenic response similar to addition of angiogenic factors [94].

While angiogenic factors can induce MMP expression in endothelial and stromal cells, MMPs can enhance the availability/bioactivity of angiogenic factors. Degradation of ECM releases ECM/basement membrane-sequestered angiogenic factors, VEGF, bFGF, and TGF β [8]. MMP-1 and MMP-3 degrade perlecan in endothelial-cell basement membranes to release bFGF [95]. Connective tissue growth factor (CTGF) forms an inactive complex with $VEGF^{165}$ and cleavage of CTGF by MMP-1, -3, -7, or -13 releases active $VEGF^{165}$ [96]. MMP-2, MMP-3, and MMP-7 degrade the ECM proteoglycan decorin releasing latent TGF 1, while MMP-2 and MMP-9 cleave the latency-associated peptide to activate TGF β1 [97, 98].

Proangiogenic roles of MMPs in tumor angiogenesis

In tumor angiogenesis, MMP-2 and MMP-9 have been shown to be critical for the "angiogenic switch" when tumors (or often, preneoplastic lesions) first become vascularized. In a transgenic mouse model of pancreatic islet tumorigenesis, the angiogenic switch occurs in a subset of hyperplastic islets with histological hallmarks of carcinoma *in situ* [6]. Expression of both MMP-2 and MMP-9 was up-regulated in angiogenic islets compared with preangiogenic islets, but using MMP knockout mice, it was determined that MMP-9 expression was required for the angiogenic switch, while MMP-2 contributed to tumor growth [99]. In this model, VEGF was expressed constitutively in the pancreatic islets and addition of exogenous MMP-9 to isolated islets caused a 2-fold increase in VEGF released into the medium [99]. Thus, it was concluded that the up-regulation of MMP-9 expression in angiogenic islets results in release of VEGF from the ECM that is then responsible for induction of the angiogenic phenotype [99]. Similarly, forced overexpression of MMP-9 in human breast cancer MCF-7 cells resulted in increased tumor angiogenesis, tumor growth, and VEGF/VEGFR-2 complex formation, which led the authors to hypothesize that MMP-9 controls release of VEGF from the ECM [100]. In a rat chondrosarcoma model, angiogenic tumor nodules produced three times the amount of MMP-2 as compared to avascular nodules, and down-regulation of MMP-2 expression by antisense oligonucleotides resulted in loss of angiogenic potential and inhibited tumor growth [101]. Thus, in this model, MMP-2 appears to be the important regulator of the angiogenic switch. In other studies, implantation of tumor cells into either MMP-2 or MMP-9 knockout mice have demonstrated reduced

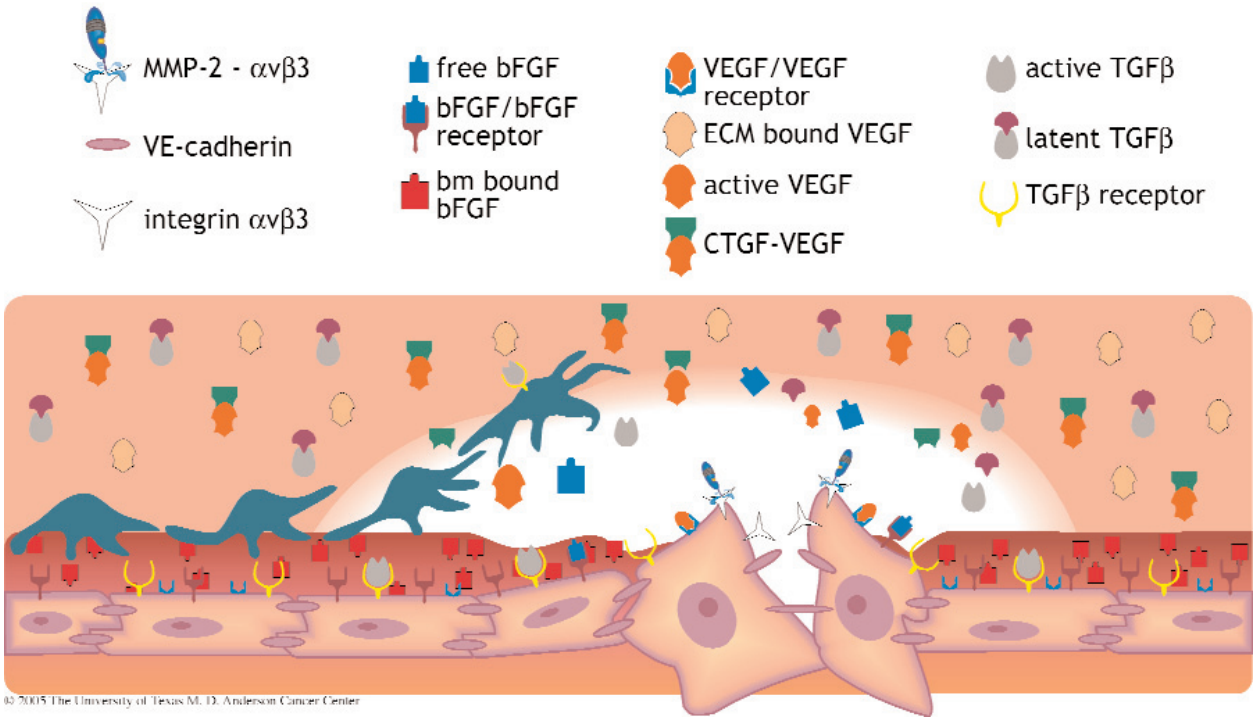

Fig. 3A Pro- and antiangiogenic actions of MMPs. A. Proangiogenic effects of MMPs include degradation of vascular basement membrane and ECM components to allow detachment of pericytes and endothelial cell migration; cleavage of VE-cadherin endothelial cell-cell adhesions; cell-surface localization of MMP-2 to the invading edge of migrating endothelial cells *via* integrin αvβ3 binding; exposure of cryptic integrin binding sites from cleaved ECM molecules; release of active VEGF from ECM- and connective tissue growth factor (CTGF)-bound stores; cleavage of basement membrane perlecan to release bFGF; and release and activation of TGF β. Bioactive VEGF, bFGF, and TGF β induce angiogenesis by signaling through their respective receptors on endothelial cells.

tumor-induced angiogenesis compared to implantation into wild type mice [102, 103]. In blood vessels within tumors of human neuroblastoma cells implanted in MMP-9 knockout mice, there were reduced numbers of pericytes, suggesting that MMP-9 is needed for recruitment of pericytes to new vessels [103].

MMPs are generally abundantly expressed in virtually all human cancers. High expression levels of certain MMPs, either by the tumor cells themselves, by stromal fibroblasts, or by infiltrating inflammatory cells, are correlated with tumor invasive and/or metastatic potential and poor prognosis [42]. Cancer cells can induce tumor stromal cells to express MMPs *via* paracrine secretion of cytokines and growth factors [61]. Co-culture of non-small cell lung cancer cells with fibroblasts induced expression of MMP-11 and bFGF by the fibroblasts [104]. Tumor expression of MMP-1, -2, -3, -7, -9, -13, and MT1-MMP in particular have been correlated with invasion capacity, metastasis, and/or risk of recurrence [42].

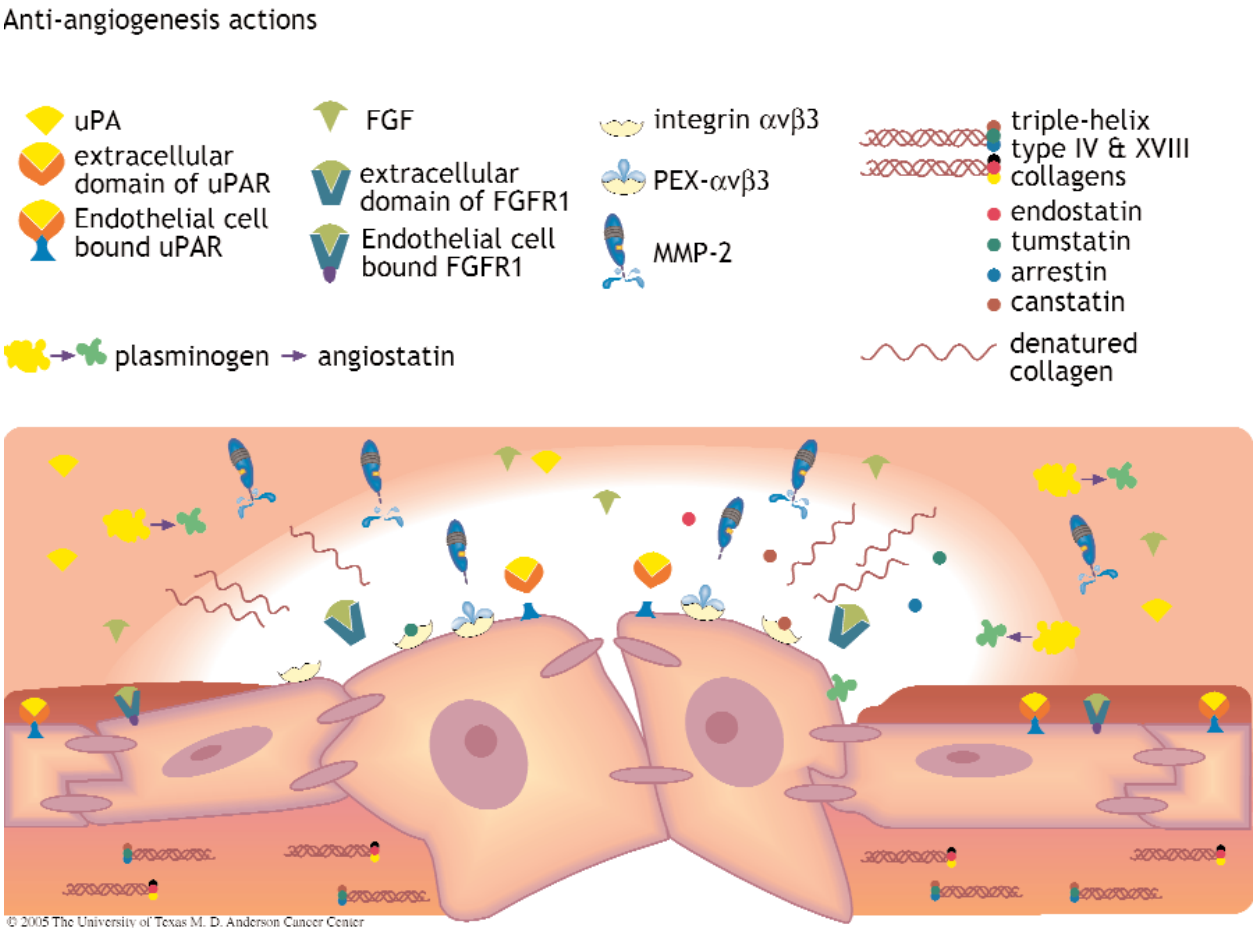

Fig. 3B Pro- and antiangiogenic actions of MMPs. Antiangiogenic effects of MMPs include cleavage of ligand-binding domains of FGFR1 and uPAR inhibiting FGFR1 signaling and uPA signaling/localization to endothelial cell surface, respectively; inhibition of MMP-2 binding to αvβ3 integrin by release of MMP-2 soluble PEX domain; and generation of antiangiogenic factors, angiostatin from plasminogen and endostatin, tumstatin, arrestin, and canstatin from noncollagenous 1 (NC1) domains of type XVIII and IV collagen chains.

Cancer cells can also stimulate angiogenesis directly by the expression and secretion of angiogenic growth factors. For example, gliomas overexpress bFGF, while Kaposi's sarcoma spindle cells can be induced to express and release bFGF by TNF α, IL-1, and interferon-γ [86]. In the mouse skin carcinogenesis model, activation of the proto-oncogene H-*ras* is a critical event in tumor initiation, as well as for induction of VEGF expression and angiogenesis [105]. Endothelioma cells were found to release chemotactic factors for endothelial cells that stimulated the secretion of latent and active MMP-1, MMP-2, and MMP-9 from endothelial cells, which correlated with increased invasiveness of the endothelial cells [106]. In ovarian carcinoma cells, which express MMP-2 and MMP-9, addition of exogenous active MMP-2 or MMP-9 induced the release of VEGF, suggesting that tumor cell-expressed MMPs can act in an autocrine manner to induce secretion of angiogenic factors [107].

MMPs are also involved in another aspect of tumor angiogenesis, that is vascular mimicry, where

tumors cells act like endothelial cells, expressing endothelial cell-associated genes and forming fluid-conducting channels within tumors. MMP-2, MT1-MMP, and laminin5γ2, which are overexpressed in aggressive melanomas, are responsible for these tumor cells' ability to undergo vascular mimicry [36]. In this system, phosphoinositide 3-kinase was found to be responsible for the up-regulation of MT1-MMP, which regulates MMP-2 activation and so, laminin5γ2 chain cleavage to promigratory fragments [36, 108]. Specific inhibition of phosphoinositide 3-kinase decreased MMP-2 and MT1-MMP activities, blocked cleavage of laminin5γ2 chain, and abrogated melanoma vasculogenic mimicry [108].

Antiangiogenic roles of MMPs

Not all MMP activities are proangiogenic, however. Fig. 3B illustrates some of the antiangiogenic activities of MMPs. MMP-2 has been shown to cleave the ectodomain of FGFR1, which retains FGF binding activity, but is unable to signal, and so, modulates the biological availability, mitogenic, and angiogenic activities of FGFs [51]. Similarly, MMP-12 cleaves uPA receptor (uPAR), releasing the uPA binding domain, which reduces functional uPAR signaling and inhibits angiogenesis [109]. Degradation of MMP-2 can result in the appearance of its soluble PEX domain, which through binding to integrin αvβ3 blocks MMP-2 binding to αvβ3, angiogenesis, and tumor growth [83]. Naturally occurring PEX has been found in conjunction with αvβ3 in tumors and during developmental retinal neovascularization [83]. In addition, MMPs have been shown to generate endogenous angiogenesis inhibitors by proteolytic cleavage of plasma proteins and ECM components. MMP-2, MMP-7, MMP-9, and MMP-12 all have the capacity to hydrolyze plasminogen to form the potent angiogenesis inhibitor, angiostatin [110–112]. Angiostatin inhibits endothelial cell migration, proliferation, and tube formation, and induces apoptosis [11]. Integrin α1 knockout mice have elevated levels of MMP-7 and MMP-9 (in skin explants and endothelial cells, and in plasma of tumor-bearing animals), and angiostatin (in plasma) [113]. Tumor cells implanted in α1 knockout mice have reduced tumor angiogenesis compared to tumors grown in wild type mice [113]. Reducing MMP-9 levels by pharmacological methods in either wild type or α1 knockout mice, resulted in reduced angiostatin levels and increased tumor growth and vascularization [78]. Conversely, stable overexpression of MMP-9 in a mouse colon carcinoma cell line resulted in increased angiostatin levels and decreased tumor growth and angiogenesis *in vivo* [78].

Other angiogenesis inhibitors can be generated by MMPs from ECM components. The C-terminal noncollagenous 1 (NC1) domain of several collagen chains, generated by proteolytic cleavage, show antiangiogenic activity [8]. These include endostatin (the NC1 domain of type XVIII collagen α1 chain), tumstatin (type IV collagen α3 chain NC1), arrestin (type IV collagen α1 chain NC1) and canstatin (type IV collagen α2 chain NC1) [8]. Endostatin can be released by MMP-3, MMP-9, MMP-12, MMP-13, and MMP-20, as well as by several cathepsins [114]. Endostatin binds to cell surface proteoglycans, to VEGFR-2, and to integrin α5β1 (RGD-dependent) to inhibit VEGF and bFGF-induced endothelial cell migration and to induce apoptosis [8, 115]. In addition, endostatin blocks the activation and activity of MMP-2, MMP-9, MMP-13, and MT1-MMP [116, 117]. Tumstatin, which can be released by MMP-9, inhibits endothelial cell proliferation and promotes apoptosis *via* signaling through integrin αvβ3 in an RGD-independent manner [115, 118]. Decreased levels of tumstatin in MMP-9 knockout mice was shown to be responsible for increased Lewis lung carcinoma growth in these mice compared to wild type mice [118]. Arrestin binds to integrin α1β1, while canstatin binds both αvβ3 and α3β1, which presumably mediates their antiangiogenic activities [8]. The NC1 domain of type IV collagen α6 chain and a fragment of fibronectin, anastellin, have also been shown to have antiangiogenic activity, although whether these two fragments can be generated by MMPs has not been demonstrated [8, 119].

Antiangiogenic thrombospondins have been shown to regulate MMP expression and activity. At low concentrations, TSP-1 was shown to induce MMP-9 secretion, tube formation, and invasive activity by endothelial cells, while higher concentrations induced higher levels of MMP-9, but reduced tube formation and invasion [120]. In a mammary tumorigenesis model (due to mouse mammary tumor virus-directed *neu* expression),

tumor burden and angiogenesis were increased in TSP-1 knockout mice due to increased levels of active MMP-9 and increased VEGF/VEGFR-2 interaction [121]. *In vitro*, TSP-1 was shown to inhibit activation of MMP-9 by MMP-3 or trypsin [121]. On the other hand, fibroblasts from TSP-2 knockout mice have elevated levels of MMP-2 and defective adhesion [122]. TSP-2 physically interacts with MMP-2, which results in low density lipoprotein receptor-related protein-mediated endocytosis of MMP-2 by fibroblasts [70].

Inhibition of MMP activity/activation as antiangiogenesis therapy

Because of the important role of MMPs in angiogenesis, MMP inhibitors have been used to block pathological angiogenesis. Endogenous MMP inhibitors, the TIMPs, have been shown to have antiangiogenic activities. TIMP-2 inhibits bFGF-induced endothelial cell proliferation, TIMP-3 inhibits cell migration and proliferation of stimulated endothelial cells, and TIMP-4 inhibits endothelial cell tube formation in the basement membrane extract, Matrigel [123]. TIMP-1 inhibits basal and VEGF-induced endothelial cell migration, which is directly dependent on MMP inhibition [124]. However, in other studies, overexpression of TIMP-1 was found to increase VEGF expression in mammary carcinoma cells and enhance VEGF-induced neovascularization in the retina [123]. Some of TIMP-2 antiangiogenic effects are not directly due to its MMP inhibitory activity, as a mutant TIMP-2, incapable of inhibiting MMPs, suppressed VEGF-induced endothelial cell migration and induced RECK expression as well as wild type TIMP-2 [124]. Cathepsin B, found at sites of angiogenesis in cancer and osteoarthritis, was shown to be able to fragment and inactivate TIMP-1 and TIMP-2, and so, stimulate angiogenesis [125].

Synthetic MMP inhibitors (MMPIs) have also shown antiangiogenic activity and some are in clinical development. First generation MMPIs were based on the collagen-peptide backbone containing a zinc-binding hydroxamate moiety, which inhibits enzymatic activity [126]. N-Biphenyl sulfonyl-phenylalanine hydroxiamic acid (BHPA), a potent inhibitor of MMP-2, MMP-9, and MT1-MMP (IC50s, 12–17 nM), was shown to strongly inhibit tumor (HT1080 fibrosarcoma)-induced angiogenesis *in vivo*, while an inactive enantiomer of BHPA had no effect [127]. This MMPI also inhibited primary tumor growth and liver metastasis using different cancer cell lines [127]. Similarly, a different peptide-hydoxamic acid-based MMPI, KB-R7785, which inhibits MMP-1, MMP-3, and MMP-9 (IC50s, 2–4 nM), was shown to be a potent inhibitor of murine C-26 colon adenocarcinoma-induced angiogenesis, primary tumor growth, and experimental lung metastasis [128]. Other MMPIs that have demonstrated antiangiogenesis activity in vivo include another peptidomimetic MMPI (batimastat or BB-94), nonpeptidic MMPIs (prinomastat or AG3340 and tanomastat or BAY 12-9566), which are based on the 3-dimensional conformation of the MMP zinc-binding site, a nonantimicrobial tetracycline analog (metastat or Col-3), which inhibits both MMP synthesis and activities, and shark cartilage extract (neovastat or Æ-941), which inhibits the activity of MMP-2, MMP-9, MMP-12, and MMP-13, as well as the function of VEGFR-2 [42]. BMS-275291, an orally bioavailable, sulfhydryl-based MMPI that inhibits MMP-1, MMP-2, MMP-7, MMP-9, and MT1-MMP (IC50s, 9–157 nM) inhibited VEGF/bFGF-induced *in vivo* angiogenesis (Matrigel plug assay), as well as inhibiting B16BL6 melanoma experimental lung metastasis [129]. A Phase I clinical trial of BMS-275291 has already been done, which used a wound angiogenesis assay to measure the effect of this MMPI on angiogenesis in humans [130]. A 4 mm punch biopsy wound on the forearm of each patient was made before and after 14-days of oral treatment with BMS-275291 and the vascularization of the wound was monitored over 2 weeks, with the before-treatment wound angiogenesis for each patient serving as the control for comparison after treatment. A delay in wound angiogenesis was detected in this assay after MMPI treatment [130]. This was the first MMPI clinical trial to use angiogenesis as an endpoint and demonstrated that MMPIs can inhibit physiological wound angiogenesis in humans as well as pathological tumor-induced angiogenesis in animals.

Initial clinical trials using MMPIs as cancer treatments did not demonstrate efficacy in terms of reducing tumor progression partially due to the fact

that most trials were done in patients with advanced stage disease, when the tumor vasculature is already well-established, and to the fact that MMPs play multiple roles in both angiogenesis and tumor progression [126]. In addition, broad spectrum MMPIs can inhibit the ADAMTSs, which have antiangiogenic activity [126]. More specific MMPIs are now being designed and tested. In addition, monoclonal antibodies to MT1-MMP, which inhibit its enzymatic activity and so activation of proMMP-2, have been shown to inhibit endothelial cell migration and invasion of collagen and fibrin gels [92]. Another approach to inhibiting proMMP-2 activation is the use of recombinant PEX domain. Lentiviral delivery of PEX to endothelial cells inhibited their invasion and tube formation in Matrigel *in vitro* and delivery of PEX to the chick chorioallantoic membrane inhibited bFGF-induced and tumor-induced angiogenesis *in vivo* [131].

Conclusion

MMPs are crucial for angiogenesis, being necessary to degrade the ECM to make room for migrating endothelial cells. MMPs also contribute to angiogenesis by detaching pericytes from vessels undergoing angiogenesis, by releasing ECM-bound angiogenic growth factors, by localizing on the cell surface of the invading tips of migrating endothelial cells, by exposing cryptic, proangiogenic integrin binding sites in the ECM, by generating promigratory ECM fragments, and by cleaving VE-cadherin to break endothelial cell-cell adhesions. Thus, clinical use of compounds that inhibit the activity or activation of MMPs is attractive for inhibiting pathological angiogenesis. However, the MMPs also have antiangiogenic activities including modulating cell surface receptor signaling by cleaving off the ligand-binding domain of FGFR-1 and uPAR, and by generating angiogenesis inhibitors from ECM components, plasminogen, and the PEX domain from MMP-2. Therefore, the use of broad-spectrum MMP inhibitors could also inhibit the antiangiogenic activities of MMPs and so, enhance angiogenesis. Caution must then be exercised before use of MMPIs as therapies for pathological angiogenesis, and much more preclinical work is needed to verify that candidate therapies will indeed inhibit angiogenesis in specific situations.

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