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Review

Human endothelial gelatinases and angiogenesis

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Abstract

Endothelial cell invasion is an essential event during angiogenesis (formation of new blood vessels). The process involves the degradation of the basement membrane and the underlying interstitium. The matrix metalloproteinase (MMP) family is considered to be primarily responsible for matrix degradation. Two members of the family, gelatinase A and B play an important role in angiogenesis. This review outlines recent findings on their regulation in human endothelial cells. Latent gelatinase B is secreted from endothelial cells. This enzyme can also accumulate in the cytosol as an active enzyme, free of TIMP-1. In contrast, latent galatinase A is constitutively secreted from the cells. Unlike other MMPs, gelatinase A activation occurs on the cell membrane and is mediated by MT1-MMP. A number of physiological activators have recently been described. These include thrombin and activated protein C, both of which activate gelatinase A independent of the MT1-MMP pathway. These new findings may lead to therapeutic interventions for the treatment of angiogenic-dependent diseases such as cancer and arthritis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Human endothelial cells; Gelatinases; Type I collagen matrix; Membrane-type matrix metalloproteinase; Thrombin

Contents

1. Introduction 961
2. Matrix metalloproteinases (MMPs) 962
3. Gelatinase are vital during angiogenesis 962
4. Human gelatinase B 963

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5. Regulation of gelatinase B in endothelial cells 963

6. Human gelatinase A 964

7. Regulation of gelatinase A activation in endothelial cells 965

8. Current model of the role of gelatinases in angiogenesis 966

9. Conclusions and future considerations 967

References 967

1. Introduction

Angiogenesis is a process by which new blood vessels develop from the existing microvascular bed. Physiological angiogenesis, which occurs in reproduction, placental development and wound repair, usually occurs in short bursts and is self-limiting. In contrast, pathological angiogenesis, which occurs in a number of diseases such as solid tumours and rheumatoid arthritis, often persists indefinitely [1,2]. Folkman was the first to propose that vascularization of tumour growth is essential for its survival [3].

Angiogenesis occurs by a series of sequential steps. In response to angiogenic stimuli, endothelial cells that line the existing microvessels degrade their basement membrane by secreting proteolytic enzymes including the matrix metalloproteinases (MMPs) and serine proteases [4]. The cells then migrate through the degraded basement membrane and continue to break down the interstitial stroma as they move. The migrating endothelial cells at the tip of sprout do not usually divide, whereas the trailing cells at the base of the new vessel undergo proliferation. The endothelium then aligns in a bipolar fashion to form a lumen. The newly formed hollow sprouts anastomose with each other to form a capillary through which, blood flows.

Recent findings have indicated that MMPs, particularly gelatinase A and B, play a central role during angiogenesis. Some characteristics of these enzymes are shown in Table 1. This review highlights recent advances in the regulation of these two enzymes in human endothelial cells as well as their role in angiogenesis.

Table 1
Characteristics of the gelatinases

Common names	Gelatinase A, 72 kD gelatinase	Gelatinase B, 92 kD gelatinase
Nomenclature	MMP2	MMP9
Substrate specificity	Gelatin, type I, IV, V collagen, elastin	Gelatin, type I, IV, V collagen, elastin
Molecular mass	72 kD	92 kD
Molecular mass of active species	64 kD, 62 kD	82 kD, 67 kD
Physiological activators	MT1-MMP, type 1 collagen, lipopolysacharride, hepatocyte growth factor, thrombin, APC	Serine proteases
Latent form binds to TIMP	TIMP-2	TIMP-1
Synthesis by endothelial cells	Constitutive	Inducible

2. Matrix metalloproteinases (MMPs)

MMPs are a family of enzymes that play a central role in ECM turnover and remodelling based on their ability to hydrolyze major protein components of the ECM [5]. They are secreted as inactive proenzymes that require activation by the removal of the propeptide, revealing the Zn-binding active site. MMPs are active at neutral pH and require Ca^{2+} for full activity. They are specifically inhibited by the tissue inhibitors of MMPs (TIMPs). There are four TIMPs, of which, TIMP-1 appears to be the most ubiquitous. The regulation of MMP activity occurs at many stages including gene activation and transcription, translation and secretion of latent enzyme, proenzyme activation and inactivation by endogeneous inhibitors. In normal physiology, MMP activity is associated with various processes such as ovulation [6], trophoblast invasion [7], skeletal development [8], mammary gland involution [9] and angiogenesis [10]. Loss of control of MMP activity has been implicated in a number of diseases such as rheumatoid arthritis and osteoarthritis [11], tumour invasion [2], and degradation of the myelin-basic protein in neuroinflammatory diseases [12].

There are currently more than 20 MMPs, which differ in their substrate specificity but share a number of common structural and functional similarities [13]. The most recent MMP identified is MMP-26 [14]. All MMPs have been assigned an MMP number and most also have a common name. They contain at least three domains: a signal peptide that directs the translational product to the endoplasmic reticulum for secretion, a propeptide domain that is removed when the enzyme is activated and a catalytic domain. Most MMPs also possess a C-terminal domain with sequence homology to hemopexin. Gelatinase A (MMP2) and gelatinase B (MMP9) differ from other MMPs in that, the catalytic domain is separated from the hemopexin-like domain by a fibronectin-like domain. Gelatinase B also has a collagen-like domain [15,16]. The membrane-type MMPs (MT-MMPs) contain a transmembrane domain that is responsible for anchoring the enzyme onto the plasma membrane [17–19].

The propeptide domain consists of approximately 80–90 amino acids. It contains a cysteine residue that is involved in activation and a highly conserved sequence PRCGVPD. The function of the propeptide domain is to maintain latency of the proenzyme. The catalytic domain contains two zinc ions and a calcium ion that are involved in the catalytic process. The C-terminal hemopexin-like domain plays an important role in substrate binding and/or in interaction with TIMPs. For example, the binding of this domain with the triple helical interstitial collagen is essential for its cleavage by collagenase [20], whilst the hemopexin domain of gelatinase A is required for an interaction with TIMP-2 during the cell surface activation of the enzyme [21,22].

MMPs are vital during angiogenesis. Montesano et al. [23] demonstrated that endothelial tube formation within collagen gel is blocked by the MMP inhibitor, 1,10-phenanthroline. Moses et al. [24] purified a protein from cartilage that inhibits bFGF-induced endothelial cell migration and proliferation in vitro, as well as angiogenesis in the CAM assay. This cartilage-derived inhibitor was later found to inhibit collagenase. Fisher et al. [25] reported inhibition of PMA-induced endothelial cell invasion and tube formation within collagen gel when treated with TIMP-1 or with the hydroxyamate-based synthetic inhibitor, BB-94. TIMP-1 and TIMP-3 have also been shown to prevent endothelial cell migration and tube formation induced by bFGF [26,27]. Haas et al. [28] showed that when rat endothelial cells are cultured for 4 days in a three-dimensional type I collagen matrix, they organize into tubular networks, a process that was reversed by the addition of the MMP inhibitor, marimastat.

3. Gelatinase are vital during angiogenesis

Both gelatinase A and B are well known for their ability to degrade collagens present in the vascular basement membrane [29]. The gelatinases also assist the collagenases in the degradation of the interstitium. Gelatinase A can breakdown the interstitial components by directly cleaving type I collagen at a rate similar to that of interstitial

collagenase [30]. Crabbe et al. [31] have reported that active gelatinase A can promote the activation of latent interstitial collagenase. The latter cleaves the triple helix of type I, II and III collagens at the Gly^{775}–Leu^{776} peptide bond, generating three-quarters and one-quarter fragments. These denatured fragments can then be further degraded by active gelatinase A and/or gelatinase B [29].

Recent in vitro and in vivo studies have specifically implicated a role for the gelatinases in angiogenesis. When endothelial cells are cultured on Matrigel, the formation of tubular networks is increased by the addition of recombinant gelatinase A and decreased when neutralizing antibody or TIMP-2 is added [26]. Itoh et al. [32] have reported a substantial reduction in angiogenic activity as well as in subsequent tumour progression in gelatinase A-deficient mice. Using the CAM system, Brooks et al. [33] demonatrated that a fragment of gelatinase A, which comprises the C-terminal hemopexin-like domain, termed PEX, significantly attenuates angiogenesis. This fragment is thought to compete with active gelatinase A for binding to the integrin $\alpha_v\beta_3$ on the cell membrane, thus blocking cell surface proteolytic activity [34]. Fang et al. [35] used a tumour nodule model to demonstrate that gelatinase A is required for the tumour to switch to the angiogenic phenotype. In this model, inhibition of gelatinase A activity resulted in a suppression of tumour growth by 70%. Koivunen et al. [36] demonstrated that a synthetic peptide, CTTHWGFTLC, inhibits endothelial migration in vitro and prevents tumour growth in mice, without any apparent toxicity effect. Interestingly, this peptide specifically inhibits both gelatinase A and B and has no effect on several other members of the MMP family or serine proteases. A role for gelatinase B in angiogenesis has been further demonstrated in gelatinase B-deficient mice [37], which exhibit abnormal skeletal growth plate vascularization and ossification.

4. Human gelatinase B

Human gelatinase B is synthesised as a polypeptide of M_r of 78 426 [38]. The proenzyme has a M_r of 92 kD, but migrates as an 88 kD protein under non-reducing conditions on SDS-polyacrylamide gel. The secreted gelatinase B is heavily glycosylated due to the presence of three N-linked glycosylation sites and several O-linked glycosylation sites, thus accounting for the extra mass of the mature enzyme. The structure of the human gelatinase B gene has been determined by Huhtala et al. [39]. The promoter region contains two putative TPA responsive elements (TRE) that may serve as the binding sites for the transcription factor AP-1. There is also a consensus sequence of a transforming growth factor-β inhibitory element.

5. Regulation of gelatinase B in endothelial cells

Human progelatinase B is secreted from endothelial cells in response to the tumour promoting chemical, phorbol myristate acetate (PMA) [40]. There have been few reports on the effects of cytokines or angiogenic/growth factors on gelatinase B secretion by human endothelial cells. Hanemaaijer et al. [41] have shown that TNF-α can enhance the effect of PMA, but it does not stimulate gelatinase B synthesis by endothelial cells when used alone. Marc et al. [42] reported that activated CD40 T cells interact with the endothelial nerve growth factor receptor, CD40, on saphenous vein endothelial cells to stimulate gelatinase B production by the latter.

Gelatinase B was thought to be synthesised and immediately secreted as a latent form. However, we have reported an accumulation of both the latent and active forms of gelatinase B in the cytosol of human endothelial microvascular cells in response to PMA [43]. Immunogold electron microscopy revealed that gelatinase B was localised in secretory vesicles. Interestingly, cytosolic gelatinase B was free of TIMP-1, whereas gelatinase B secreted was bound to TIMP-1, suggesting that the formation of gelatinase B and TIMP-1 occurred after their secretion. Gelatinase B was also localised to the endothelial cell membrane, possibly binding to the hyaluronan receptor, CD 44 [44].

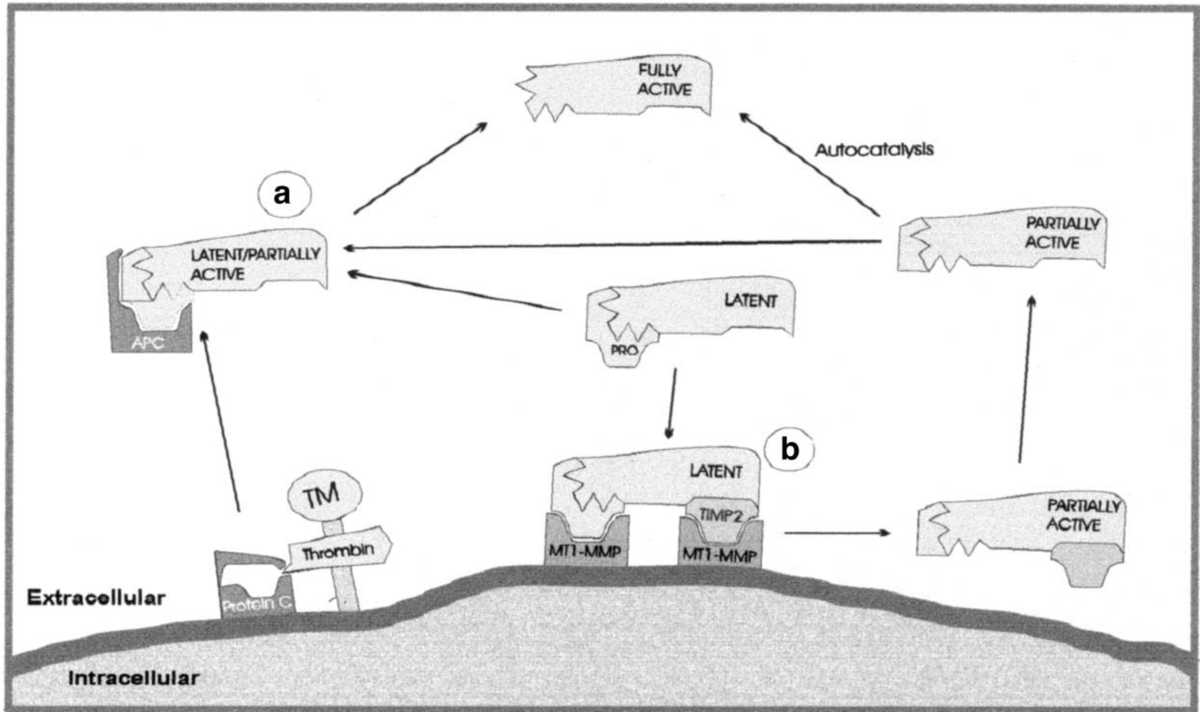

Fig. 1. Activation gelatinase A on the surface of endothelial cells. At least two separate mechanisms are known to activate gelatinase A on the endothelial surface: (a) APC, Thrombin binds to thrombomodulin on the endothelial surface and activates protein C to form APC. APC directly activates the latent and/or partially active gelatinase A to the fully active form; (b) MT1-MMP, TIMP-2 binds to the catalytic domain of active MT1-MMP via its N-terminal domain. Latent gelatinase A interacts with TIMP-2 via its C-terminal domain forming a trimolecular complex. A nearby free MT1-MMP then partially activates progelatinase A by initiating a cleavage in the propeptide domain of progelatinase A. The partially active gelatinase A is released and subsequently autocatalysed to the fully active form.

6. Human gelatinase A

Human gelatinase A has a M_r of 72 kD (migrates as a 66 kD protein under non-reducing conditions) on SDS-polyacrylamide gel, in agreement with its amino acid sequence. Although gelatinase A has a similar substrate specificity to that of gelatinase B, it is differently regulated at both transcriptional and extracellular levels [45]. Unlike other MMPs, the promoter of gelatinase A gene lacks the TRE sequence as well as the known transactivator sequences, AP-1 and PEA-3 [46]. This may explain the lack of gelatinase A upregulation by agents such as PMA, TNF-α or Il-1. In addition, the promoter region of gelatinase A has a unique noncanonical TATA box, which may be responsible for basal secretion of the enzyme. Another distinct feature is the absence of an upstream TGF-β inhibitory element in the promoter which is consistent with the observation that gelatinase A transcription is not suppressed by TGF-β [46].

Gelatinase A is unique among the MMPs in that it does not possess the propeptide sequences that are susceptible to proteolytic activation by other proteases such as plasmin or trypsin. Moreover, unlike most other MMPs where activation occurs in the extracellular milieu, activation of gelatinase A can occur on the cell membrane via

the membrane-type MMP (MT1-MMP). The current model of progelatinase A activation by MT1-MMP is shown in Fig. 1 and explained as follows: MT1-MMP is synthesised as a 63 kD latent enzyme, which is intracellularly processed to the 60 kD active enzyme and transported to the cell membrane. TIMP-2 binds to the catalytic domain of active MT1-MMP via its N-terminal domain. Progelatinase A interacts with TIMP-2 via its C-terminal domain forming a trimolecular complex. A nearby free MT1-MMP then activates progelatinase A by initiating a cleavage in the propeptide domain of progelatinase A generating the 62 kD intermediate form. The partially active species is further processed over time to the 59 kD fully active form by autocatalysis [47,48]. TIMP-2 allows the activation to occur as long as its concentration does not exceed the binding capacity of MT1-MMP. The partially active gelatinase A-TIMP-2 complex may be released from the membrane or remains bound to the cell membrane via MT1-MMP or the integrin $\alpha_v\beta_3$ [34,49].

7. Regulation of gelatinase A activation in endothelial cells

Human endothelial cells constitutively secrete latent gelatinase A. The expression of the latent enzyme is not upregulated by PMA [50,51], although Hanemaaijer et al. [41] have shown that stimulation of umbilical vein endothelial cells (HUVE) with PMA resulted in the secretion of the two active forms (64 and 62 kD) of gelatinase A. Similarly, progelayinase A activation induced by PMA has been reported in neonatal foreskin microvascular endothelial cells [52]. Lewalle et al. [50] demonstrated the presence of both the latent and the two active forms of gelatinase A in the membrane fraction of HUVE when stimulated with PMA. Foda et al. [51] reported that MT1-MMP expression was upregulated when treated with PMA. Activation of gelatinase A was sensitive to TIMP-2, but not TIMP-1. Thus, it appears that PMA-induced progelatinase A activation in human endothelial cells follows the same pathway that is dependent on the proteolytic activity of MT1-MMP, as previously reported in other cell types.

Haas et al. [28] reported that a three-dimensional matrix of type I collagen activates progelatinase A in rat capillary endothelial cells, and co-ordinately increases MT1-MMP expression. Recently, we have demonstrated a similar effect in human endothelial cells and confirmed that collagen activates gelatinase A via the MT1-MMP pathway [53]. Furthermore, activation of gelatinase A by collagen is delayed, but sustained for long periods of time in culture. The ability of type 1 collagen to activate gelatinase A suggests that endothelial cells may use active gelatinase A to move through the type I collagen-rich interstitium during angiogenesis.Zucker et al. [54] have demonstrated that thrombin induces gelatinase A activation in human endothelial cells. We recently reported that thrombin-induced activation differs from that of type 1 collagen [55]. Whereas collagen exhibits a delayed effect, thrombin activates gelatinase A rapidly, within 2 h. The thrombin protease-activated receptors are not required for the activation. This is evidenced by the finding that the synthetic thrombin receptor activating peptide is unable to activate gelatinase A [54]. Activation by thrombin, however, requires an endothelial membrane molecule, but interestingly, it does not involve MT1-MMP. Thus, gelatinase A activation induced by thrombin operates via a novel pathway that is independent of the thrombin receptor and proteolytic activity of MT1-MMP.

During the anticoagulation process, thrombin triggers the protein C pathway by interacting with thrombomodulin (TM) which is constitutively expressed on the surface of endothelial cells. The thrombin–TM complex converts protein C to the anticoagulant serine protease, activated protein C (APC) [56,57]. Recently, we have shown that APC activates gelatinase A in human endothelial cells [58]. Like thrombin, APC-induced activation is rapid and does not require MT1-MMP. APC activation of gelatinase A occurs in the absence of cells, indicating that it acts directly. Recent data from our laboratory suggests that thrombin activates gelatinase A through APC, by binding to thrombomodulin and activating protein C (unpublished observations) (Fig. 1).

Other stimulators of gelatinase A activation in human endothelial cells include sublethal levels of hydrogen peroxide [59], hepatocyte growth factor [60], lipopolysaccharide [61] and ligation of the integrin CD40 on EC with either membranes from activated $CD4^{+}$ T cells or human recombinant CD40 ligand [42]. Rajavashith et al. [62] have reported that treatment of human saphenous vein endothelial cells with TNF-α, IL-α or oxidized low density lipoprotein upregulates MT1-MMP which, results in the activation of gelatinase A.

8. Current model of the role of gelatinases in angiogenesis

Based on recent findings, a model to explain the role of gelatinases during angiogenesis, in diseases such as cancer and arthritis, is described below and is depicted diagrammatically in Fig. 2. Endothelial cells continually secrete latent gelatinase A under basal conditions in vitro. In vivo, gelatinase A has been found to be strongly expressed by some endothelial cell types, including in human glioblastomas [63]. Thrombin, which is present at high levels in angiogenic situations such as cancer [64,65] and rheumatoid arthritis [66], interacts with thrombomodulin on the endothelial surface and activates protein C. APC then rapidly activates latent gelatinase A, causing disruption of existing capillary bed. The effect of thrombin is likely to be short-lived as it is readily incorporated into fibrin clots, immobilized in the subendothelial basement membrane or inactivated by agents such as antithrombin III, protein C inhibitor, heparin co-factor II or heparin [67]. In addition to APC,

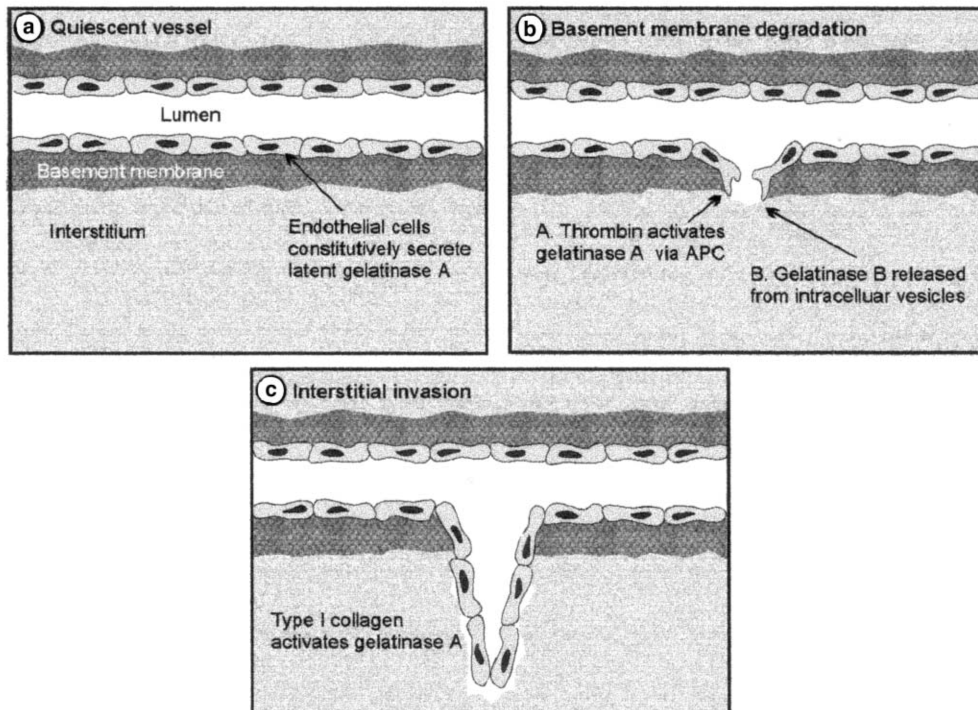

Fig. 2. Role of gelatinases during angiogeneis: (a) Gelatinase A is constitutively secreted by endothelial cells; (b) Thrombin, which is elevated in angiogenic situations, activates protein C on the endothelial surface. The resultant APC rapidly activates gelatinase A allowing the endothelial cells to degrade the basement membrane. Active gelatinase B may be released from intracellular storage vesicles of endothelial cells to assist basement membrane degradation; (c) Once the endothelial cells have penetrated the basement membrane they contact the interstitium. Type I collagen further activates gelatinase A for sustained periods of time.

other angiogenic agents such as hydrogen peroxide and hepatocyte growth factor may contribute to the activation of gelatinase A. As endothelial cells migrate during this initial phase of angiogenesis, gelatinase B may participate in the degradation of the basement membrane. It is feasible that gelatinase B is secreted from the storage vesicles within the cell, in short bursts, to locally degrade the basement membrane.

The gelatinases are rapidly inactivated by TIMPs, which bind to their catalytic domain [68,69]. Alternatively, free enzyme can be degraded to inactive low M_r species by autocatalysis [70]. These inhibitory mechanisms are important, as they prevent uncontrolled proteolysis. Pepper et al. [71] have shown that if serine proteolysis goes uninterrupted, the dissolution of the matrix prevents endothelial cells from migrating and forming tube-like structures. This occurs due to impairment of endothelial cell adhesion and the disruption of the cell-matrix interactions that are required for cell migration.

After breaking down the basement membrane, endothelial cells make contact with type I collagen, which is the predominant protein in the interstitial stroma. Type I collagen matrix upregulates MT1-MMP and activates gelatinase A. Activation of gelatinase A by type I collagen can be sustained for long periods of time in vitro. This is likely due to the fact that, unlike thrombin and APC, type I collagen can be biologically active indefinitely as long as the interactions between the integrins and collagen are maintained. The long duration of collagen-induced activation of gelatinase A may at least partly explain the extended duration of angiogenesis in diseases such as cancer and rheumatoid arthritis. Activation of gelatinase A is likely to persist until the newly formed capillary secretes its basement membrane.The latter then acts as a barrier to prevent further contact with type I collagen. In addition to gelatinase A, other MMPs are likely to play a role especially, the collagenases and MT-MMPs. Thus, the presence of type I collagen in the interstitial stroma could perpetuate its own degradation by endothelial cells, allowing for endothelial migration and subsequent angiogenesis.

9. Conclusions and future considerations

It has become apparent in recent years that gelatinases are vital for angiogenesis. The recent discoveries revealing mechanisms of gelatinase regulation in human endothelial cells have greatly contributed to the understanding of their role in angiogenesis. However, a number of questions remain unanswered. What triggers the release of gelatinase B from secretory vessels in the endothelial cell? How is intracellular gelatinase B activated? Of the two known pathways involved in the activation of endothelial gelatinase A, which is the most important, MT1-MMP or APC? Do these pathways cooperate in generating active gelatinase A or do they act independently in different physiological/pathological settings? The answers to these questions may have clinical implications with regards to the treatment of angiogenic diseases, such as cancer and arthritis.

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