MT1-MMP-mediated Cleavage of Decorin in Corneal Angiogenesis

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Abstract

Background/Aims—Decorin has been shown to have anti-angiogenic properties. In this study, we evaluate the involvement of membrane type 1-matrix metalloproteinase (MT1-MMP), a pro-angiogenic enzyme, in decorin cleavage in the cornea.

Methods—MT1-MMP expression was confirmed immunohistochemically in keratocytes and immortalized corneal fibroblast cell lines. Corneal micropockets of bFGF were used to assess the expression of decorin and MT1-MMP. Western blotting was used to evaluate decorin degradation by MT1-MMP. Aortic ring tube formation assays were used to assay the inhibitory effect of decorin and stimulatory effect of MT1-MMP on vascular endothelial cells *in vitro*.

Results—We show that MT1-MMP expression is upregulated following bFGF pellet implantation in the cornea *in vivo,* and that MT1-MMP cleaves decorin in a time- and concentration-dependent manner *in vitro*. Furthermore, the addition of MT1-MMP reduces the inhibitory effects of decorin on aortic ring tube formation *in vitro*. Cleavage of decorin by MT1-MMP-deficient corneal cell lysates is diminished relative to that by wild-type corneal cell lysates, and an MT1-MMP knock-in restores decorin processing *in vitro*.

Conclusion—The pro-angiogenic role of MT1-MMP in the cornea may be mediated, in part, by facilitated cleavage of corneal decorin.

Keywords

Introduction

Angiogenesis plays a critical role in a variety of pathological conditions such as tumor growth and metastasis, diabetic retinopathy, and wound healing [1,2]. Identification of the factors that contribute to the physiology of angiogenic pathways and their diseases is the subject of extensive research. Several angiogenic factors, such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), angiopoietin-2, and platelet-

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derived growth factor (PDGF) are implicated in the activation of endothelial cells [3]. Similarly, the extracellular matrix (ECM) and the matrix metalloproteinases (MMPs) also may contribute to angiogenesis and vascular development [4,5].

MMPs, a family of Zn^{2+}-dependent enzymes, are produced as latent proenzymes that may be inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) [6–9]. The MMPs are classified into two subfamilies according to structure: membrane-type (MT-MMP) and secreted MMPs [10]. Of the six MT-MMPs described thus far, MT1-MMP possesses broad-spectrum activity and cleaves various ECM components such as type I and II collagens, fibronectin, vitronectin, laminin, fibrin, and proteoglycan [11–14].

Recent evidence suggests that MT1-MMP may play an important role in cellular invasion by disrupting ECM barriers [15–18]. Furthermore, MT1-MMP participates in the proteolytic degradation of ECM components such as the basement membrane or interstitial stroma associated with angiogenesis [19,20]. For example, Li *et al.* found that MT1-MMP degrades lumican and abrogates lumican-mediated suppression of tumor cell colony formation in soft agar [21]. Abd El-Aziz *et al.* demonstrated that MT1-MMP contributes to tumor cell proliferation through the cleavage of growth differentiation factor 15 (GDF15), a transforming growth factor (TGF)-beta superfamily member [22]. Endothelial cells from lungs of one-week-old MMP-14 knockout (MMP14$^{-/-}$) mice show reduced migration and limited ability to form three-dimensional structures on matrigel [17,18,23–25].

Corneal lumican, decorin, and keratocan, are involved in corneal morphogenesis that modulate collagen fibrillogenesis [26]. Decorin belongs to a family of structurally-related proteoglycans, grouped as the small leucine-rich proteoglycans, and is a normal constituent of the corneal stroma [27]. Decorin has been shown to inhibit vascular endothelial cells migration and tube formation when endothelial cells are grown on decorin-coated surfaces [28]. Sulochana et al. demonstrated that purified decorin and the leucine-rich repeat 5 (LRR5) of decorin core protein function as angiogenesis inhibitors by inhibiting both vascular endothelial growth factor (VEGF)- and basic fibroblast growth factor (bFGF)-induced angiogenesis [29]. Decorin binds to insulin-like growth factor I (IGF-I) receptor in a corneal inflammatory angiogenesis model [30], and IGF-I receptor is up-regulated in decorin-deficient mice [31]. The addition of recombinant decorin to wild-type tumor cells caused 80–95% suppression of VEGF mRNA and protein, and resulted in decreased *in vivo* tumor growth [32].

Previous *in situ* hybridization studies demonstrated the expression of MT1-MMP in corneal keratocytes and epithelial cells in unwounded and keratectomy-wounded corneas [33]. We also demonstrated that MT1-MMP regulates angiogenesis in part by cleaving Type XVIII collagen in the cornea [33]. A major component of the corneal stroma is decorin, which is a potent inhibitor of angiogenesis. In this study, we investigated the effects of MT1-MMP on decorin processing and degradation, and demonstrated that the degradation of decorin by MT1-MMP, either directly or indirectly, results in a pro-angiogenic effect in the cornea.

Materials and Methods

Animals and Antisera

All animal studies were conducted in accordance with the Animal Care and Use Committee guidelines of the Massachusetts Eye and Ear Infirmary and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 wild-type mice were used for *in vivo* experiments. Rabbit polyclonal anti-MT1-MMP antisera preparations (designated C14) that recognize the cytoplasmic domain (QRSLLDKV) of MT1-MMP were generated in-house in established protocols.

MT1-MMP knockout mice were kindly provided by Drs. Zhou and Tryggvason (Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

Corneal Micropocket Procedure

Mouse corneal micropocket procedures were performed as previously described [34,35]. Mice were anesthetized using a combination of ketamine and xylazine injections. Supplemental proparacaine eye drops were used for local anesthesia. Corneal micropockets were created using a modified von Graefe knife in wild-type mice. Hydron pellets (0.4×0.4 mm) containing 120 ng of human recombinant bFGF (R&D Systems Inc., Minneapolis, MN) were implanted into the corneal pockets. Ofloxacin eye drops were instilled after surgery. The eyes were photographed by slit lamp microscopy (Nikon, Tokyo, Japan) and enucleated for immunohistochemistry and western blot analysis on postoperative day seven.

Western blotting

Unwounded wild-type mouse corneas and corneas that were implanted with pellets were excised on day seven. The cells were lysed in a buffer containing 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, and 50 mM Tris-HCl [pH 7.6], mixed with a sample buffer containing β-mertcaptoethanol, boiled for 2 min, and subjected to western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using 4–20% Tris-Glycine gradient gels (Invitrogen, Carlsbad, CA). After SDS-PAGE, proteins were transferred electrophoretically to a hydrophobic polyvinylidene difluoride membrane (Immobilon-P, Millipore Co., Bedford, MA). The membrane was incubated at room temperature (RT) for 1 hr in $1\times$ TBST (Tris-Buffered Saline Tween-20) containing 3% BSA (Sigma-Aldrich, St. Louis, MO), and then incubated at RT for 1 hr in TBST containing primary antibody (goat anti-mouse decorin antibody, 1:500, R&D Systems Inc. or rabbit anti-MT1-MMP polyclonal antisera, 1:1000). The membrane was then incubated at RT with shaking for 30 min in TBST containing the second antibody (horseradish peroxidase-conjugated donkey anti-goat IgG, 1:20,000, Pierce, Rockford, IL, or donkey anti-rabbit IgG, 1:20,000, Amersham Biosciences, England). Each step was followed by extensive washing in TBST. Subsequently, the membrane was washed three times for 10 min at RT with $1\times$ TBST. Antigen detection was achieved by incubation of the membrane for 1 min at RT with a chemiluminescent substrate (SuperSignal West Pico; Pierce) and exposure to X-Omat Blue XB-1 Film (Kodak Inc., Rochester, NY) for 10 sec or, if necessary, up to 30 min. For quantification, films were digitally scanned, and band densities were measured quantitatively using Scion Image software (version 4.0.3, Scion Corp., Frederick, MD). The band densities were expressed as ratios of the mean band density from experimental samples to that obtained from normal mouse corneas.

Confocal Immunohistochemistry

Mouse corneas were embedded in OCT compound (Tissue-Tek, Miles Laboratories, Naperville, IL) at −20°C. Frozen sections were cut at a thickness of 8 μm, placed on microscope slides, air dried, and then fixed in 4% paraformaldehyde for 5 min. After blocking with 3% BSA in PBS, sections were incubated at RT for 2 hr with specific primary antibodies diluted in BSA/TBST. Either goat anti-mouse decorin antibody (1:100) or rabbit anti-MT1-MMP (C14) antisera (1:100) were used for staining. After washing with PBS, sections were incubated at RT for 30 min with FITC-conjugated anti-goat or anti-rabbit IgG secondary antibodies (1:400, both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Stained sections were observed by confocal microscopy (TCS 4D; Leica, Heidelberg, Germany). Propidium iodide (Vector Laboratories, Burlingame, CA) was used for nuclear staining.

Aortic Ring Assays

Aortic ring assays were performed as described [36]. Briefly, aortas were obtained from wild-type mice, and fatty tissues around the aorta were removed carefully under a surgical microscope. A one millimeter-long aortic ring was cut and rinsed in five consecutive washes of EBM (Cambrex, Walkersville, MD). Wells of 48-well plates were coated with 150 µl of rat tail type I collagen (BD Biosciences, Bedford, MA). After gelling at 37°C for 30 min, an aortic ring was placed on its side on top of the gel and sealed in place with an overlay of 100 µl of collagen. One hr later, 300 µl of EBM containing 20 ng/ml purified decorin (Sigma-Aldrich) and/or 20 ng/ml recombinant human MT1-MMP (Calbiochem, NJ) was added to each well. On days six and nine, the gels were photographed with a phase contrast microscope equipped with a digital spot camera (Micro Video Instruments, Avon, MA). Tube formation was determined by counting the number of microvessels and analyzing according to reported criteria [36].

Immortalized Corneal Keratocyte Cell Lines

Immortalized mouse corneal cell lines from wild-type (WT) mice were generated as described previously [37] and immunostained with anti-vimentin antibodies. Briefly, corneal keratocytes were isolated by incubating MT1-MMP knockout mouse corneas, immortalized with SV40T large T antigen and subcloned via serial dilutions. To generate MT1-MMP knock-in cell lines, MT1-MMP cDNA construct was subcloned into the pFB vector under CMV promoter following with Intra ribosomal entry sequence (IRES) and EGFP coding sequence. Recombinant viruses were generated by transfection of 293T cells with the pFB vector encoding MT1-MMP or an empty control vector using Effectene™ transfection reagent (Qiagen, Valencia, CA). MT1-MMP containing virus was generated and used to infect MT1-MMP knockout keratocyte cell lines. The medium obtained from the second and third days after transfection, which has higher virus expression, was collected and used for the infection of corneal cell lines. The corneal keratocyte cell line from WT or MT1-MMP KO mice were infected with control or MT1-MMP virus, and the cells were analyzed by flow cytometry on the Coulter EPICS XL-MCL flow cytometer (Coulter Electronics Inc., Miami, FL). Stained cells were sorted using a Coulter ELITE cell sorter into EGFP-positive and EGFP-negative cells. The stability of the expression of EGFP was monitored by flow cytometry.

EGFP positive cells were then plated at a concentration of 10,000 cells/ml in DMEM, supplemented with 10% heat-inactivated FCS at 37°C in 5% CO_2. DMEM containing 0.5% FCS was used for starvation conditions. When the cells reached 80% confluence, the cells were kept in the serum-free medium. After 24 hours, cells were collected and lysed in 1ml of RIPA buffer [50mM Tris/HCl (pH 7.5)/150mM NaCl/1% (v/v) Nonidet P-40/0.25% (w/v) sodium deoxycholate] and incubated at 4°C for 30 min. The purpose of the 30-minute incubation was to solubilize the cellular protein.

Cell Lysate-mediated Decorin Cleavage Assay

All cleavage assays were carried out in a volume of 50 µl. 100 ng Decorin was incubated in reaction buffer (60 mM Tris-HCl [pH 6.5], 150 mM NaCl, 25 µM $ZnSO_4$, and 25 µM $CaCl_2$) with corneal keratocyte cell lysates obtained from cultured WT, MT1-MMP KO, KO/MT1-MMP mouse keratocytes. RIPA buffer was used as the control. The degradation pattern of decorin was analyzed by gel electrophoresis and western blot using anti-decorin antibodies (R&D Systems, Inc.).

Data analysis

Statistical analyses were performed using the SPSS program (Chicago, IL). Results were expressed as mean ± SEM. Data was analyzed using one-way analysis of variance (ANOVA), and a value of p<0.05 was considered statistically significant.

Results

Expression of MT1-MMP in bFGF-induced corneal neovascularization

To examine the expression of MT1-MMP, the C14 anti-MT1-MMP antisera, which recognizes the cytoplasmic domain of MT1-MMP, was used to stain normal and vascularized mouse corneas. Seven days following bFGF pellet implantation into mouse corneas to induce neovascularization, immunohistochemistry revealed positive reactivity against MT1-MMP in the stroma (Fig. 1). MT1-MMP expression was detected in bFGF treated corneas (Fig. 1B). The specificity of the C14 MT1-MMP antisera was verified by preabsorption of cognate peptide antigen, and little to no detectable MT1-MMP immunostaining was present in the vascularized cornea (Figure 1E). Western blot analysis using the C14 antisera revealed strong bands in the corneas that was 58 kDa in size (Fig. 1H). The density of 58 kDa immunoreactive bands was significantly higher in the vascularized corneas (N = 4) than in the normal corneas (N = 4, $p < 0.05$; Fig. 1H).

Expression and cleavage of decorin in bFGF-induced corneal neovascularization

Because decorin is known to be a potent inhibitor of angiogenesis and is a major component of the corneal stroma, the expression of decorin was analyzed in normal and vascularized corneas using immunohistochemistry, and cleavage was examined using western blot analysis. In the normal mouse cornea, decorin was expressed throughout the entire depth of the stroma, as expected (Fig. 2 A–C). However, in bFGF pellet-induced vascularized corneas, decorin was not detected around corneal vessels and pellet areas (Fig. 2 D–F).

The degradation pattern of decorin was compared between normal and vascularized corneas using western blot analysis (Fig. 2J). Presumed decorin degradation products were noted as lower molecular weight fragments in vascularized corneas and were not detected in normal corneas (Fig. 2J).

Cleavage of decorin by MT1-MMP

To analyze the cleavage of decorin by MT1-MMP, the two proteins were incubated under various experimental conditions *in vitro*. Western blot analysis indicated that decorin was cleaved by MT1-MMP in the presence of Zn^{2+} and Ca^{2+}, as little to no MT1-MMP-mediated cleavage of decorin was observed in the absence of Zn^{2+} and Ca^{2+} (Fig. 3A). Decorin proteolysis by MT1-MMP was more efficient at pH 5.5 and pH 6.5 when compared to that at pH 7.6 (Fig. 3B). The kinetics of decorin proteolytic processing by MT1-MMP occurred in a concentration- and time-dependent manner (Fig. 3C and 3D, respectively). Minimal cleavage was observed after 1 hr, and complete cleavage of the substrate required almost 20 hrs (Fig. 3D). GM6001 inhibited MT1-MMP cleavage of recombinant decorin (Fig. 3E).

Aortic ring tube formation inhibition by decorin, and reversal by the addition of recombinant active MT1-MMP

To assess the proangiogenic activity of MT1-MMP *in vitro* in the presence of decorin, mouse aortic rings embedded in collagen gel were maintained in EBM containing MT1-MMP and/or decorin. The *in vitro* sprouting of microvessels in the aortic ring assay at days six and nine was inhibited by media containing decorin (Figs. 4B and E, respectively). When

MT1-MMP was added to decorin-containing media, the number of microvessel tubes was increased at days six and nine (Figs. 4C and F, respectively), suggesting that the pro-angiogenic effect of MT1-MMP offsets the anti-angiogenic effect of decorin (Fig. 4G).

Cleavage of decorin by cell lysates or culture medium obtained from cultured keratocytes

MT1-MMP mRNA was determined by real time PCR in WT-Ker, KO-ker, and KO-ker/ MT1-MMP cell lines (Fig. 5A). To further examine decorin proteolysis, the MT1-MMP-mediated cleavage of decorin was assayed in the presence of cell lysates from mouse corneal keratocytes. The addition of cell lysates from WT keratocytes (Fig. 5B) enhanced the cleavage of decorin to generate decorin degradation products. Reduced amounts of decorin degradation fragments (25–35kDa) were observed following addition of cell lysates from cultured MT1-MMP KO keratocytes (Fig. 5B). Decorin degradation was re-established in the presence of lysates from MT1-MMP knock-in keratocytes (Fig. 5B). These results suggest that MT1-MMP derived from WT and knock-in keratocytes may contribute to decorin degradation.

Discussion

Our current findings support the hypothesis that the pro-angiogenic effect of corneal MT1-MMP may be mediated, in part, by the cleavage of an anti-angiogenic factor, decorin. Other possible pro-angiogenic mechanisms of MT1-MMP include: 1) the production of angiogenic factors, such as VEGF; 2) the degradation of anti-angiogenic factors in addition to decorin; and/or 3) the breakdown of extracellular matrix components to facilitate vascular endothelial cell migration. These mechanisms could occur either directly or via MMP-2 activation, as we previously reported that corneal angiogenesis is diminished upon bFGF stimulation in mice deficient in MMP-2, a substrate of MT1-MMP [35]. Alternatively, additional substrates of MT1-MMP may exist to play a role. Tam *et al.* (2004) used membrane proteomics to characterize several potential MT1-MMP substrates [38]. These substrates include extracellular matrix proteins and signaling molecules such as fibronectin, laminin, type I collagen, MMP-2, and -13, TNF-alpha and CTGF [38]. Additionally, Abd El-Aziz *et al.* (2007) have shown that MT1-MMP contributes to tumor cell proliferation through the cleavage of GDF15, which down-regulates cell proliferation [22].

Although mice deficient in other MMPs show minimal apparent phenotypic changes, MT1-MMP-deficient mice exhibit an extremely disfigured phenotype due to inadequate collagen turnover and marked deceleration of postnatal growth, which leads to death by approximately three weeks of age. Additionally, these mice display a deficiency in the ability to induce corneal neovascularization after bFGF-pellet implantation [39]. Due to the lack of appropriate anti-mouse MT1-MMP antibodies, characterization of the temporal and spatial expression patterns of MT1-MMP, and assessment of MT1-MMP involvement during bFGF pellet implantation was difficult. In this study, we successfully generated polyclonal antisera to a discrete region of mouse MT1-MMP, specifically to the cytoplasmic (C14) domain. It is not clear whether the 58 kDa band recognized by the antisera represents truncated or active MT1-MMP. However, it is likely that the 79 kDa band represents full length Pro-MT1-MMP. Additional studies are needed to further clarify these questions.

The mouse corneal model is well suited for characterizing pro-angiogenic and anti-angiogenic responses, given that the mouse cornea contains several well characterized ECM molecules with known anti-angiogenic properties, including collagen XVIII, collagen IV, decorin, and thrombospondin. We have shown previously that collagen XVIII is proteolytically processed by MT1-MMP. Our current findings demonstrate that MT1-MMP is involved in the cleavage of decorin, a previously uncharacterized substrate, and this process may regulate corneal neovascularization. Increasing evidence suggests that decorin

possesses anti-angiogenic properties. Jarvelainen *et al.* (2006) found that decorin influenced new vessel formation in the cutaneous wound-healing model in decorin-deficient mice [40]. Additionally, Grant *et al.* (2002) demonstrated that decorin suppresses tumor cell-mediated angiogenesis by inhibiting tumor-derived VEGF production [35]. Furthermore, Davies Cde *et al.* (2001) found that decorin alone, or in combination with thrombospondin-1, inhibited angiogenesis *in vitro* [28]. Lastly, Nelimarkka *et al.* (2001) reported that decorin might play a role in inflammation-associated angiogenesis, given that capillary endothelial cells in pyogenic granuloma and granulation tissue of healing dermal wounds produced decorin [41]. However, other reports suggest that decorin promotes rather than inhibits angiogenesis. Schonherr *et al.* (2004) showed that a decorin deficiency leads to impaired angiogenesis in injured mouse corneas in a chemical cauterization model [31]. Importantly, the studies suggesting that decorin is either an inhibitor or a stimulator of angiogenesis are not necessarily contradictory, rather they indicate the molecular complexity of angiogenesis under differential physiologic and pathologic conditions.

In this report, we demonstrate bFGF-induced MT1-MMP expression and diminished decorin expression around bFGF-pellet implanted areas in vascularized mouse corneas. Additionally, we show that MT1-MMP cleaves decorin *in vitro* and that cell lysates from MT1-MMP-deficient keratocytes have lost their decorin-processing activity. Lastly, we find that purified decorin inhibits vascular tube formation in an aortic ring assay and that the addition of recombinant active MT1-MMP reverses the decorin-mediated inhibition. Although our data suggests a role for decorin in MT1-MMP-mediated angiogenesis pathways, other anti-angiogenic factors also may be involved in MT1-MMP-mediated corneal neovascularization, as previously mentioned. Based on the important role of MT1-MMP and decorin in angiogenesis, an understanding of the mechanism of MT1-MMP/ decorin signaling and regulation in angiogenesis may provide the basis for therapeutic interventions for the treatment of corneal neovascularization, and possibly other angiogenesis-related disorders.

Acknowledgments

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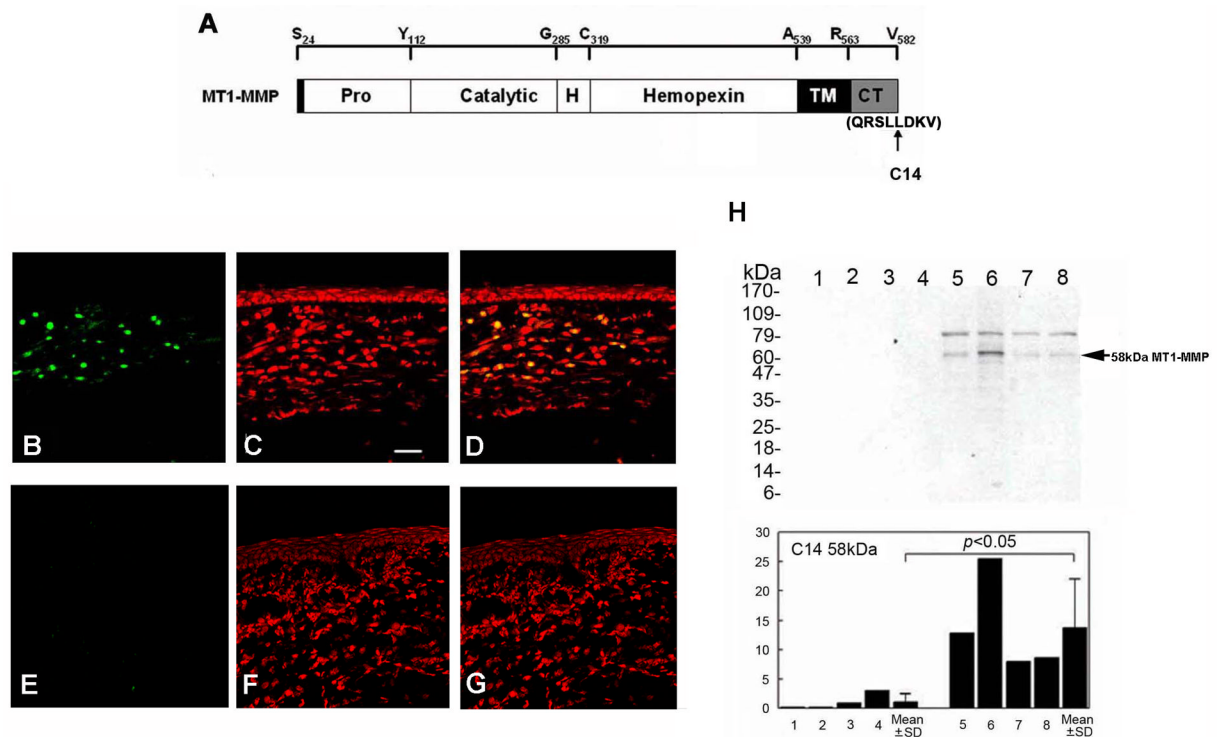
Figure 1. Enhanced MT1-MMP expression in vascularized cornea
(A) Rabbit polyclonal antisera, which recognizes the cytoplasmic domain (C14) of MT1-MMP, was generated. The schematic diagram shows the various domains of the MT1-MMP protein (Pro, prodomain; H, hinge region; TM, transmembrane domain; CT, cytoplasmic tail). (B–D) The expression of MT1-MMP was examined using immunofluorescence. The expression of MT1-MMP was examined in the mouse corneas seven days after bFGF pellet implantation and prominent staining of MT1-MMP was observed in the stroma (B). Cognate peptide competition was performed to verify the specificity of MT1-MMP antibodies (E). The second column (C, F) shows propidium iodide staining, and the third column (D, G) shows the merged image. (H) western blot analysis using anti-MT1-MMP antisera showed significantly higher levels of MT1-MMP in the vascularized corneas (N = 4, lanes 5–8) than in the normal corneas (N = 4, lanes 1–4) (upper panel). Band densities were quantified by Scion Image software (lower panels). Bar = 20μm.
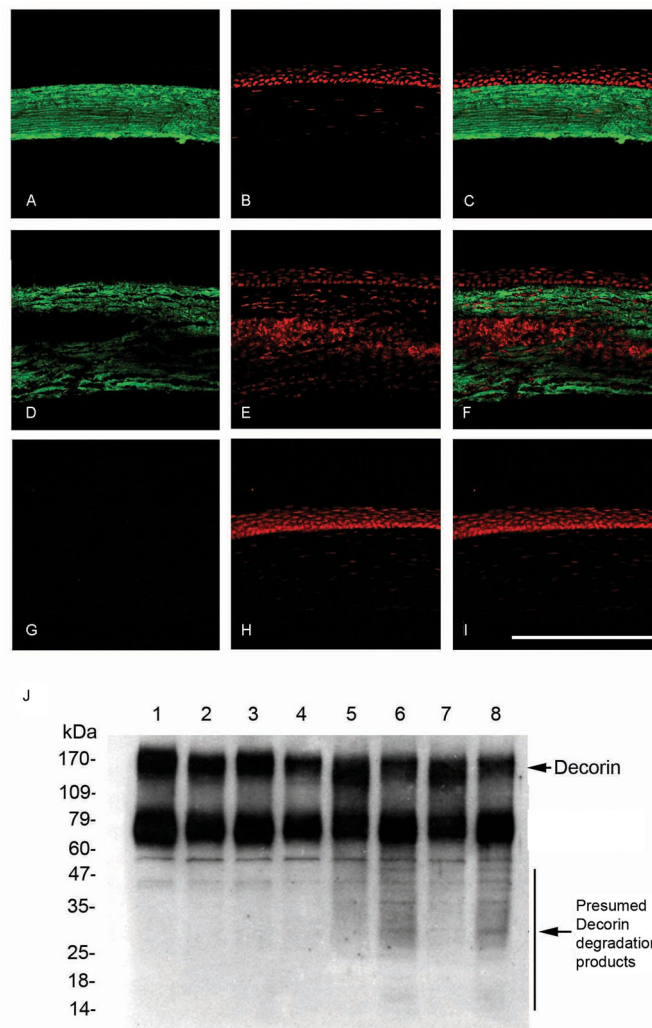
Figure 2. Expression of decorin in vascularized corneas
bFGF pellets were implanted into mouse corneas. After seven days, the corneas were sectioned and immunoblotted with anti-decorin antibodies and the expression of decorin was examined by immunofluorescence (A–I) and western blot analysis (J). The first column of the immunofluorescence analysis (A, D, G) shows staining of decorin, the second column (B, E, H) shows propidium iodide staining, and the third column (C, F, I) shows the merged image. Decorin was expressed throughout the normal corneal stroma (A). Little to no expression of decorin was detected around the pellet area in the corneal stroma (D). Primary antibodies were omitted as a negative control (G). (J) western blot analysis suggests that decorin cleavage into small fragments was greater in vascularized corneas (N = 4, lanes 5–8), as compared to normal corneas (N = 4, lanes 1–4).
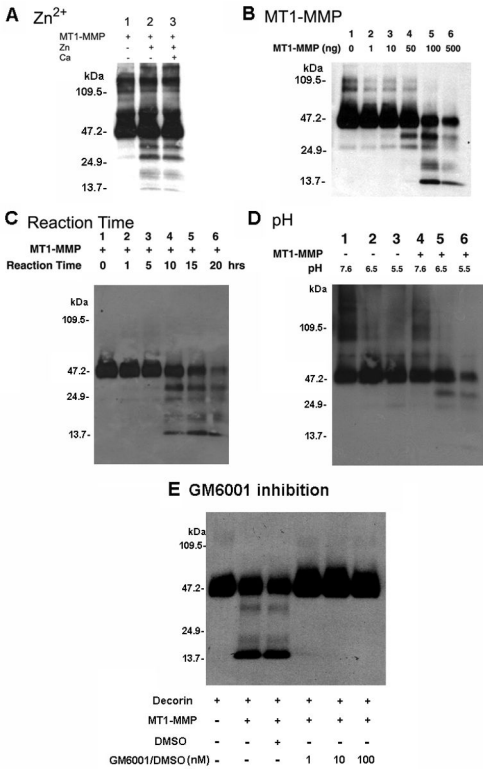
Figure 3. *In vitro* cleavage of decorin by MT1-MMP
Decorin was incubated with various concentrations of human recombinant MT1-MMP at different time points (from 0 to 20 hrs), with differential Zn^{2+} and Ca^{2+} concentrations and levels of pH. The degradation pattern of decorin was analyzed by western blot analysis. Decorin was processed by MT1-MMP *in vitro* in the presence of Zn^{2+} and Ca^{2+} (A). The kinetics of decorin proteolytic processing by MT1-MMP is regulated by MT1-MMP concentrations (B) and incubation time (C). Decorin proteolysis by MT1-MMP was enhanced at pH 6.5 when compared to that of physiologic pH 7.6 (D). GM6001 inhibited MT1-MMP cleavage of recombinant decorin (E).
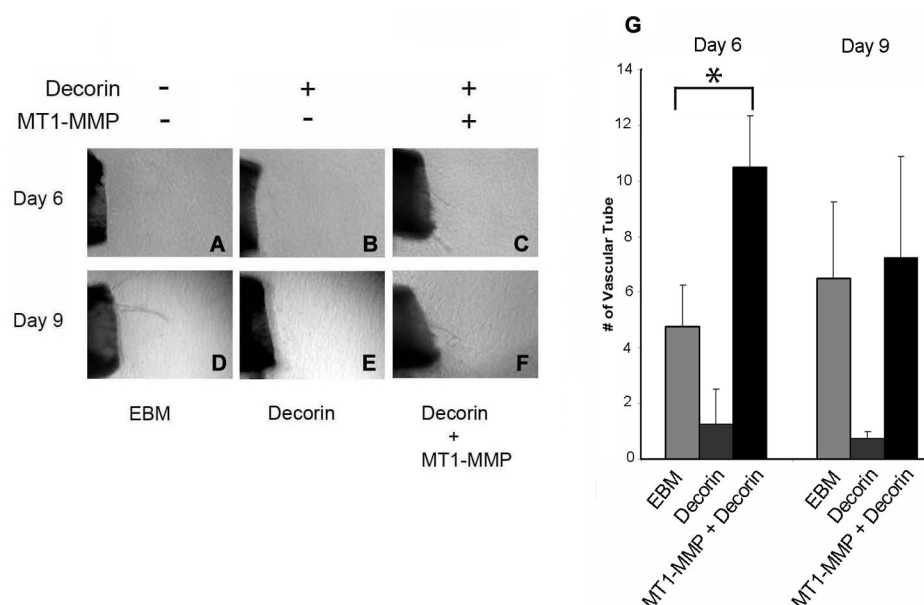
Figure 4. Enhanced vascular tube formation in the presence of decorin and recombinant MT1-MMP in an aortic ring assay

Mouse aortas were embedded in type I collagen and then bathed in complete EBM only or complete EBM supplemented with 20 ng/ml MT1-MMP and/or 20 ng/ml decorin as indicated. Photographs were taken on days 6 (A–C) and 9 (D–F) after embedding the aortas. The numbers of microvessels were counted under the microscope at each time point. Vascular tube formation was inhibited by decorin (B, E). Despite the presence of decorin, the addition of MT1-MMP diminished the inhibitory effects on microvessel tube formation mediated by decorin (C, F). Microvessels were calculated as mean ± SEM (G). * indicates statistical significance.
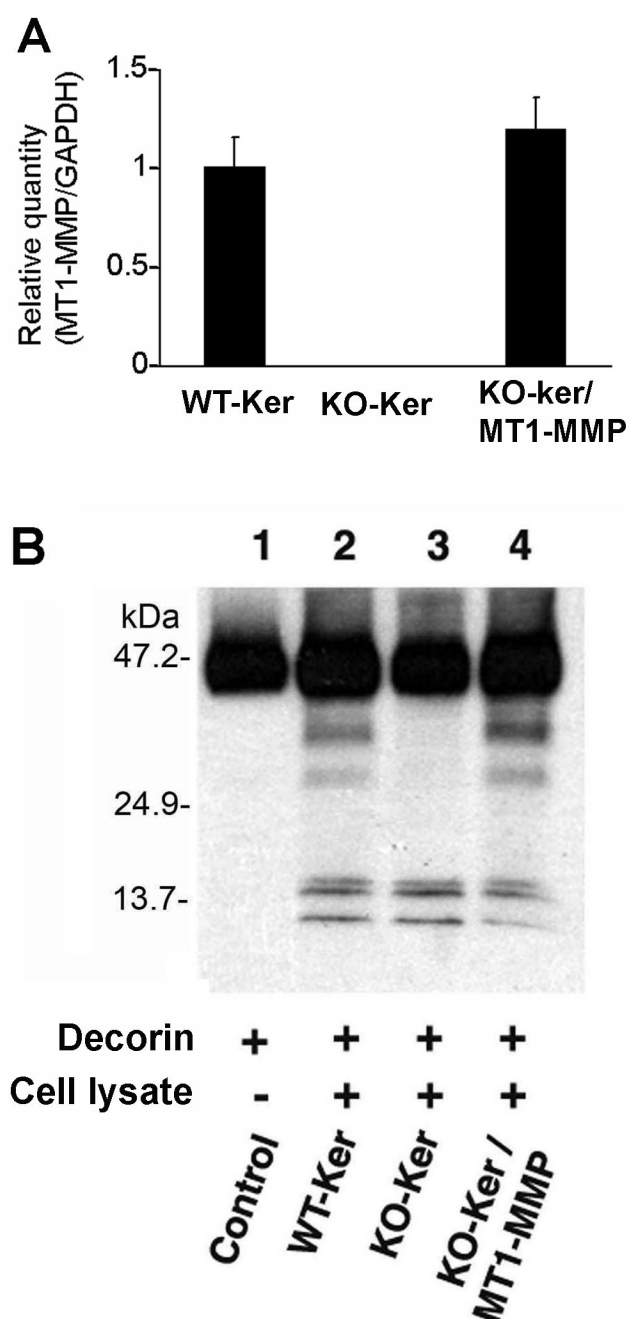
Figure 5. Decorin processed by cultured keratocyte cell lysates
Immortalized corneal cells from wild-type (WT) and MT1-MMP KO mice were subcloned
to generate corneal keratocyte cells. MT1-MMP KO cells were transfected with MT1-MMP
(KO/MT1-MMP). (A) Real time PCR showed no MT1-MMP mRNA detection in KO-ker
cells using primer from exon 4. KO-ker/MT1-MMP restored MT1-MMP mRNA expression.
(B) Cleavage of decorin was analyzed in the presence of cell lysates using western blot
analysis. Decorin was incubated with 20µl of cell lysates obtained from the various cultured
mouse keratocytes for 24 hrs. The presence of the 25–35 kDa bands show that decorin was
cleaved more efficiently by the WT (lane 2) and KO/MT1-MMP cell lysates (lane 4) than by

the KO cell lysate (lane 3). Buffer in the absence of cell lysate was used as a control (lane 1).