MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices

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Summary

Previous work has shown that endothelial cell (EC)-derived matrix metalloproteinases (MMPs) regulate regression of capillary tubes in vitro in a plasmin- and MMP-1 dependent manner. Here we report that a number of serine proteases can activate MMP-1 and cause capillary tube regression; namely plasma kallikrein, trypsin, neutrophil elastase, cathepsin G, tryptase and chymase. Plasma prekallikrein failed to induce regression without coactivators such as high molecular weight kininogen (HMWK) or coagulation Factor XII. The addition of trypsin, the neutrophil serine proteases (neutrophil elastase and cathepsin G) and the mast cell serine proteases (tryptase and chymase) each caused MMP-1 activation and collagen type I proteolysis, capillary tubular network collapse, regression and EC apoptosis. Capillary tube collapse is accompanied by collagen gel contraction, which is strongly related to the wound contraction that occurs during regression of granulation tissue in vivo. We also report that proMMP-10 protein expression is markedly induced in ECs undergoing capillary tube morphogenesis. Addition of each of the serine proteases described above led to activation of proMMP-10, which also correlated with MMP-1 activation and capillary tube regression. Treatment of ECs with MMP-1 or MMP-10 siRNA markedly delayed capillary tube regression, whereas gelatinase A (MMP-2), gelatinase B (MMP-9) and stromelysin-1 (MMP-3) siRNA-treated cells behaved in a similar manner to controls and regressed normally. Increased expression of MMP-1 or MMP-10 in ECs using recombinant adenoviral delivery markedly accelerated serine protease-induced capillary tube regression. ECs expressing increased levels of MMP-10 activated MMP-1 to a greater degree than control ECs. Thus, MMP-10–induced activation of MMP-1 correlated with tube regression and gel contraction. In summary, our work demonstrates that MMP-1 zymogen activation is mediated by multiple serine proteases and MMP-10, and that these events are central to EC-mediated collagen degradation and capillary tube regression in 3D collagen matrices.

Key words: Matrix metalloproteinase-1, Matrix metalloproteinase-10, Angiogenesis, Vascular regression, Endothelial cell, Serine protease

Introduction

Recent studies have demonstrated that proteinase-mediated degradation of the supporting extracellular matrix (ECM) is critical for vascular regression (Bajou et al., 1998; Bajou et al., 2001; Davis et al., 2001; Davis et al., 2002; Zhu et al., 2000). Elucidation of the mechanisms regulating vascular regression is essential for attempts to induce regression of angiogenic vessels associated with tumors (Browder et al., 2000; Carmeliet and Jain, 2000; Folkman, 1997; Hanahan, 1997; Holash et al., 1999) or in diseases such as diabetic retinopathy or retinopathy of prematurity (Cai and Boulton, 2002; Campochiaro and Hackett, 2003; Mechoulam and Pierce, 2003). Normally, vascular regression follows angiogenic responses during wound healing and endometrial shedding during the menstrual cycle (Ausprunk et al., 1978; Clark, 1996; Madri et al., 1996). One possibility is that the normal vascular regression response is inhibited under pathologic conditions such as that observed within a tumor wound microenvironment (Davis et al., 2002). Inhibition of vascular regression, as well as stimulation of new blood vessel formation, may together explain why tumors show increased vascularity. Vascular regression inhibitory agents include proteinase inhibitors such as plasminogen activation inhibitor-1 (PAI-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Bacharach et al., 1998; Bajou et al., 2001; Davis et al., 2001; Davis et al., 2002; Zhu et al., 2000).

Data from several laboratories suggest that extracellular matrix stability is required for a proper angiogenic response. Plasmin directly degrades fibrin, a major ECM substrate for angiogenesis (Pepper, 2001; Senger, 1996; Vernon and Sage, 1995) and further activates MMP-1, MMP-3, MMP-9 and MMP-10 (He et al., 1989; Jeffrey, 1998; Lund et al., 1999; Murphy et al., 1999; Nagase, 1998; Suzuki et al., 1990). The activated forms of these proteinases promote degradation of collagen type I, or basement membrane matrices that represent other important angiogenic substrates (Davis et al., 2002; Pepper, 2001; Senger, 1996; Vernon and Sage, 1995). Furthermore, a recent study revealed that decreased plasmin

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generation by breast carcinomas adversely affected prognosis in patients with breast cancer (Chappuis et al., 2001). These data support the hypothesis that proteolytic ECM degradation, or destruction of the angiogenic scaffold, leads to collapse of angiogenic networks.

Other studies present a different view, stating that proteinases such as plasmin and soluble MMPs stimulate angiogenic responses by promoting basement membrane degradation and ECM proteolysis necessary for EC invasion and sprouting (Carmeliet and Jain, 2000; Foda and Zucker, 2001; Pepper, 2001; Sternlicht and Werb, 2001; Werb et al., 1999; Zucker et al., 2000). Additional work has strongly implicated membrane-associated MMPs (i.e. MT-MMPs) as the major contributors to endothelial and epithelial tube morphogenesis in three-dimensions (Bayless and Davis, 2003; Davis et al., 2002; Hiraoka et al., 1998; Hotary et al., 2000). Proteinase inhibitors such as PAI-1 and TIMP-1 (which primarily block secreted proteinases) have no ability to inhibit morphogenesis, whereas inhibitors such as TIMP-2 and TIMP-3 inhibit morphogenic events (Bayless and Davis, 2003; Davis et al., 2002; Hiraoka et al., 1998). Thus, one of the issues in capillary tube morphogenesis and regression is the role of soluble versus membrane-bound MMPs. Our work concerning plasminogen and MMP-1 dependent capillary tube regression in collagen matrices in vitro suggests that soluble proteinases primarily control regression events rather than morphogenic events (Davis et al., 2001; Davis et al., 2002). This concept, recently suggested as a new mechanism for vascular regression, is very similar to findings previously described for other types of tissue regression (Curry and Osteen, 2001; Sternlicht and Werb, 2001; Werb et al., 1996; Werb et al., 1999). Two such examples include mammary gland regression (Sympson et al., 1994; Talhouk et al., 1992; Werb et al., 1996; Werb et al., 1999) and uterine endometrial shedding (Curry and Osteen, 2001; Kokorine et al., 1996; Marbaix et al., 1996). Thus, degradation of ECM is linked to regression events in the mammary gland, uterus and vasculature. Determination of the mechanism by which collagen/ECM degradation might occur is of central importance in these circumstances.

MMP zymogen activation is an essential step for the initiation of ECM proteolysis. Proper activation of the zymogen occurs after pro-peptide cleavage events (Nagase and Woessner, 1999; Sternlicht and Werb, 2001). Although plasmin has been described as a key activator of several MMPs, many other serine proteases (i.e. kallikrein, trypsin, neutrophil elastase, cathepsin G, tryptase and chymase) have also been shown to directly activate MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10 in vitro or in vivo (Duncan et al., 1998; Fang et al., 1997; Gruber et al., 1989; Nagase, 1995; Nagase, 1998; Okada et al., 1987; Sepper et al., 1997; Shamamian et al., 2001; Zhu et al., 2001). Stromelysins (i.e. MMP-3 or MMP-10) are particularly relevant in the context of capillary tube regression owing to their ability to both degrade various components of basement membrane matrix such as collagen type IV, laminin and proteoglycans (Baricos et al., 1988; Bejarano et al., 1988; Nagase, 1998) and to activate MMPs capable of degrading the interstitial matrix (MMP-1, MMP-8 and MMP-13) (Knauper et al., 1996; Nagase, 1998; Nicholson et al., 1989; Windsor et al., 1993). In addition, it is well known that the serine protease plasmin and activated stromelysins show synergistic functions in generating an active form of MMP-1 that is approximately tenfold more efficient than the enzyme generated by serine proteases alone (He et al., 1989; Suzuki et al., 1990). For these reasons, we hypothesized that a member of the stromelysin family might be involved in the capillary tube regression response.

Building on our previously described system of EC tubular morphogenesis and regression (Davis et al., 2001), we developed a quantifiable micro-well regression assay that is well suited to study ECM proteolysis in a cell-based system under serum-free conditions. Using this assay system, we report that multiple serine proteases activate MMP-1 zymogen, which leads to capillary tubular network regression in 3D collagen matrices in an MMP-1 dependent manner. Activation of EC-derived MMP-1 zymogen caused proteolysis of collagen type I, capillary tube collapse and collagen gel contraction. Additionally, we demonstrate for the first time that stromelysin-2 (MMP-10) zymogen is induced during human EC tubular morphogenesis in 3D collagen matrices and that MMP-10 pro-enzyme is activated by the above-mentioned serine proteases in a manner similar to MMP-1. We further demonstrate a direct role for both MMP-1 and MMP-10 in the regression response by inhibiting regression after transfecting ECs with MMP-1 or MMP-10 siRNAs and showing that tube regression is markedly accelerated in serine protease-treated ECs expressing increased levels of MMP-1 or MMP-10. Thus, activation of MMP-1 zymogen by multiple serine proteases and MMP-10 leads to collagen matrix degradation, EC network collapse and gel contraction. Overall, this work suggests a complementary role for MMP-1, MMP-10 and serine proteases in controlling vascular tube regression, a key event in tissue regression responses.

Materials and Methods

Plasma kallikrein, prekallikrein, factor XII and high molecular weight kininogen were obtained from Enzyme Research Laboratories (South Bend, IN). Plasminogen was obtained from American Diagnostica (Stamford, CT). Neutrophil elastase, cathepsin G, tryptase and chymase were obtained from Calbiochem (La Jolla, CA). Trypsin was obtained from Boehringer Ingelheim (Germany). The pAdEasy adenoviral system (He et al., 1998) was kindly provided by Bert Vogelstein (Johns Hopkins University College of Medicine, Baltimore, MD). Oligonucleotides were synthesized by Sigma-Genosys (Woodlands, TX). Recombinant TIMP-1 was obtained from Chemicon Corp (Temecula, CA). Recombinant PAI-1 was from Calbiochem. A monoclonal antibody directed to MMP-1 (41-1E5) used for western blotting was obtained from Oncogene Research Products (Cambridge, MA). A monoclonal antibody used to detect MMP-10 (MAB9101) was from R&D Systems (Minneapolis, MN). A monoclonal antibody used to detect MMP-3 (Clone 4B7.3, product no. M 6552) was from Sigma (St Louis, MO). Rat tail collagen type I was prepared as described (Bornstein, 1958).

Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, CA) and were cultured as described (Maciag et al., 1979). ECs were used in our experiments from passages 2-6. The standard EC morphogenesis assay in 3D collagen gels was performed as described using 3.75 mg/ml of type I collagen (Davis and Camarillo, 1996; Salazar et al., 1999). The above enzymes were added at various concentrations to the serum-free culture media consisting of Medium 199, a 1:250 dilution of the Reduced Serum supplement II, 50 μg/ml of ascorbic acid, 50 ng/ml phorbol ester, 40 ng/ml recombinant VEGF-165 (Upstate Biochemical, Lake Placid, NY) and 40 ng/ml FGF-2 (Upstate Biochemical). In the regression assays using adenoviral infected ECs, phorbol ester was not added.

To normalize viral infection, each adenovirus titer was determined with the Adeno-X Rapid Titer Kit (BD Biosciences, Palo Alto, CA). For viral infection, 1×10^6 ECs were infected with 2.2×10^7 IFU of adenovirus.

Time-lapse photography of individual cultures was performed at 4× and 10× magnification to demonstrate the capillary tube regression and gel contraction response. Cultures were fixed following gel contraction with 3% glutaraldehyde in PBS, pH 7.5 for at least 30 minutes prior to additional manipulation. In some cases, cultures were stained with 0.1% Toluidine Blue in 30% methanol and then destained prior to visualization and photography. In other cases, proteinase inhibitors were added to inhibit collagen proteolysis and gel contraction. Conditioned media were collected to examine differential proteinase expression at different times of culture. Conditioned media were run on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed and developed as described (Salazar et al., 1999). Three-dimensional collagen gels were also extracted in some cases to examine protein expression (Salazar et al., 1999) during the regression process.

384 micro-well regression assays

To objectively quantify the capillary tube regression and gel contraction response, we developed a micro-well regression assay in 384-well tissues culture plates. EC cultures were prepared as described above. For quantitative analysis of gel contraction, 15 μl mixtures of ECs and collagen matrix (n=8 cultures per condition) were placed in 384-well tissue culture plates (VWR, West Chester, PA). Cultures were allowed to equilibrate for 60 minutes prior to addition of media with or without the described amounts of serine proteases. Addition of culture media denoted time zero and cultures were monitored every 4 hours for gel contraction. Upon initiation of gel contraction, gel area was recorded using an ocular grid and the percentage of collagen gel contraction was calculated as follows: [(original area – current gel area)/original area]×100. When contraction was complete, cultures were fixed and media examined as described above. Data are reported as the mean percentage gel contraction (±s.d.).

Fluorescent collagenase assay

In order to quantify the collagenase activity of EC cultures, a commercially available, DQ collagenase assay kit was obtained (Molecular Probes, Eugene, OR). Conditioned media were collected at indicated time points and diluted 1:1 in 1× reaction buffer. For each condition and time point, triplicate samples were incubated overnight (16-20 hours) at room temperature in 25 μg/ml DQ collagen type I in black, 96-well plates. Control wells containing DQ collagen and 1× reaction buffer were incubated concurrently to determine background fluorescence. Collagenase activity was determined by measuring fluorescence in a Synergy HT fluorescent microplate reader using an excitation spectra of 485±20 nm and an emission spectra of 528±20 nm. Background fluorescence was subtracted prior to reporting of data as mean (±s.d.).

Treatment of ECs with MMP-1 siRNA for use in 384 micro-well regression assay

siGENOME SMARTpool human MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10 siRNAs were obtained from Dharmacon (Lafayette, CO). α2 macroglobulin and luciferase GL2 duplex siRNA were used as controls. Confluent ECs were split into 25cm^2 flasks at time 0. 6 hours after plating, ECs were washed and transfected with 200 nM siRNA in antibiotic-free media using 17.5 μl siPORT Amine (Ambion, Austin, TX). Five hours after transfection, cells were supplemented with 3 ml antibiotic-free growth media. After 12 hours, flasks were aspirated, supplemented with fresh, antibiotic-free growth media and allowed to recover. This transfection procedure was repeated 48 hours from the time of the first transfection. After the second transfection, cells were allowed to recover for 28 hours prior to utilization in the 384 micro-well regression assay. In experiments using both siRNA and adenoviral-mediated gene transfer, ECs were infected with adenovirus 24 hours after the second transfection and 3D cultures were established 16 hours later.

Generation of MMP-1, MMP-3 and MMP-10 adenoviruses

Recombinant adenoviruses were constructed containing the full-length cDNAs for MMP-1, MMP-3 and MMP-10. MMP-1 and MMP-3 clones were amplified by PCR from endothelial cell cDNA (MMP-1) and normal human dermal fibroblast cell cDNA (MMP-10) as described (Bell et al., 2001; Davis et al., 2001; Salazar et al., 1999). The resulting PCR products were cloned using the indicated restriction enzymes into the pAdTrack-CMV vector: MMP-1 5′: AGCTCGAGAGTATGCACAGCTTTCCTCCAC (*Xho*I) and 5′-AGAAGCTTCCATTCAAATTAGTAATGTTCAAT-3′ (*Hin*dIII); MMP-3 5′-AGAGATCTGCCACCATGAAGAGTCTTCCAATCC-3′ (*Bgl*II) and 5′-AGGATATCTCAACAATTAAGCCAGCTGTTAC (*Eco*RV).

The following primer set was utilized for the construction of full-length cDNA for MMP-10: 5′-AGAGATCTGCCACCATGATGCATCTTGCATTCC-3′ (*Bgl*II) and 5′-AGCTCGAGCTAGCAATGTAACCAGCTGTTAC-3′ (*Xho*I). The clone was amplified from ATCC vector MGC-1704, which contained the complete coding sequence for human MMP-10 zymogen. The PCR product was cloned using the indicated restriction enzymes into the pAdTrack-CMV vector.

Each pAdTrack-CMV clone was characterized by sequence analysis prior to recombination with pAdEasy-1 as described (He et al., 1998). Details of our adenoviral production protocol have been described previously (Bayless and Davis, 2002). For all constructs, protein production was verified by western blot analysis.

Results

Addition of plasma kallikrein to 3D endothelial cell cultures induces capillary tube regression and collagen gel contraction

Plasma kallikrein and plasmin have previously been shown to demonstrate a synergistic influence on inducing the regression of lactating mammary ducts (Selvarajan et al., 2001). We previously reported that the addition of plasminogen/plasmin induced MMP-1 dependent capillary tube regression in our system of 3D EC tubular morphogenesis in collagen matrices (Davis et al., 2001). To determine whether plasma kallikrein affects capillary tube regression in a similar manner to plasminogen/plasmin, a time-course experiment of kallikrein-induced capillary tube regression and collagen gel contraction was performed (Fig. 1). In this experiment, 1 μg/ml of active kallikrein was added to EC cultures as previously described (Davis et al., 2001) and the same well was photographed over time to observe the collagen gel contraction process in round, 4.5-mm-diameter micro-wells (Fig. 1A). In addition, EC cultures were established in a 384 micro-well (3-mm-square micro-wells) regression assay (Fig. 1B). Conditioned media were collected at various time points and western blot analysis of MMP-1 was performed. The capillary tube regression and collagen gel contraction response seen in Fig. 1A-B directly correlated with MMP-1 zymogen activation (Fig. 1C, arrowheads). In the absence of kallikrein, MMP-1 zymogen remained inactive and tube regression and gel contraction did not occur.

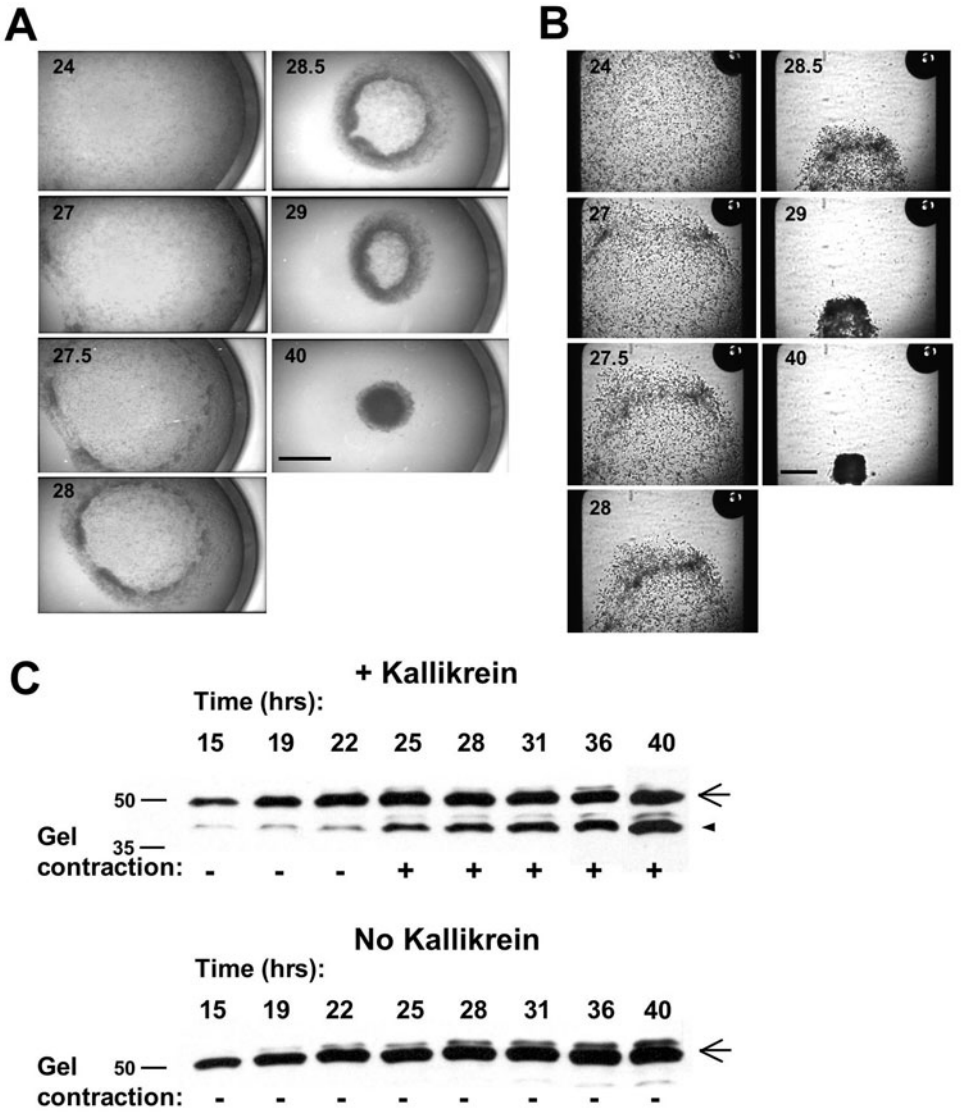

Fig. 1. Plasma kallikrein induced MMP-1 activation and contraction of endothelial tube networks. ECs were suspended in collagen matrices and plated in 96-well tissue culture plates (4.5 mm diameter) (A) or 384 micro-well plates (3 mm square) (B). Culture media contained plasma kallikrein at 1 μg/ml. The same culture well was photographed at the indicated times (in hours) during the capillary tube regression and collagen gel contraction process. (C) Conditioned media were collected and triplicate cultures were examined for evidence of capillary tube regression and collagen gel contraction (+ indicates occurrence of gel contraction). Samples were run on SDS-PAGE gels, blotted to PVDF membranes and probed with anti-MMP-1 antibodies. Arrows indicate the position of MMP-1 zymogen and arrowheads indicate activated MMP-1. Note the absence of activated MMP-1 (arrowhead) in the absence of kallikrein. Bar, 500 μm.

To clearly demonstrate the capillary tube regression phenomenon of individual EC fields, time-lapse photography was performed in the absence (Fig. 2A) or presence (Fig. 2B) of 1 μg/ml kallikrein. In the absence of kallikrein, EC vacuole, lumen and network formation occurred normally (Fig. 2A) as previously described (Davis et al., 2001; Davis and Camarillo, 1996). ECs cultured in the presence of kallikrein (Fig. 2A) underwent normal morphogenesis initially, as evidenced by EC sprouts and vacuoles (arrows). The kallikrein-induced regression response is characterized by capillary tube and vacuole collapse (arrowheads), which eventually leads to individual EC rounding, aggregation, death and capillary tube network retraction and collapse (Fig. 2B, 29, 36, 47 hours). The capillary tube regression response is further illustrated in Fig. 2C with established EC tubular networks. EC cultures were established in the absence of serine proteases and allowed to undergo tube morphogenesis and network formation for 48 hours prior to the addition of 1 μg/ml of active kallikrein. An EC-lined tube (Fig. 2B, arrows, 48.5 hours) is shown undergoing tube regression (arrowheads, 52, 52.5, 53 hours) as indicated by tube collapse prior to the onset of overall collagen gel contraction.

To further determine the concentration effects of active kallikrein during capillary tube regression, varying doses of the activated enzyme were added to ECs in the 384 micro-well regression assay (Fig. 3A). Quantification of the capillary tube regression and gel contraction response is shown as the percentage of gel contraction over time. The addition of active plasma kallikrein resulted in a marked dose- and time-dependent activation of MMP-1 zymogen that directly correlated with capillary tube collapse and gel contraction (Fig. 3A). In our previous study, plasminogen/plasmin-induced capillary tube regression likewise correlated with MMP-1 zymogen activation and cleavage of the type I collagen matrix (Davis et al., 2001). Additionally, we demonstrated in our prior study that plasminogen/plasmin-induced capillary tube regression led to time-dependent EC apoptosis, whereas ECs cultured in the absence of plasminogen did not undergo apoptosis (Davis et al., 2001). In a similar manner, the addition of kallikrein to EC cultures induced capillary tube regression, collagen gel contraction and EC apoptosis. Western blot analysis of ECs undergoing kallikrein-induced tube regression showed marked induction of active caspase-3, decreased levels of pro-caspase-3 and pro-caspase-7, and cleavage of the caspase-3 substrates gelsolin and α-fodrin compared to control cultures (data not shown). Addition of the proteinase inhibitors, aprotinin (2900 KIU/ml), TIMP-1 (1 μg/ml) and PAI-1 (2.5 μg/ml), not only completely blocked the kallikrein-induced tube regression and collagen gel contraction response (as well as the development of EC apoptosis), but also inhibited activation of MMP-1 zymogen (not shown). Thus, kallikrein-induced MMP-1

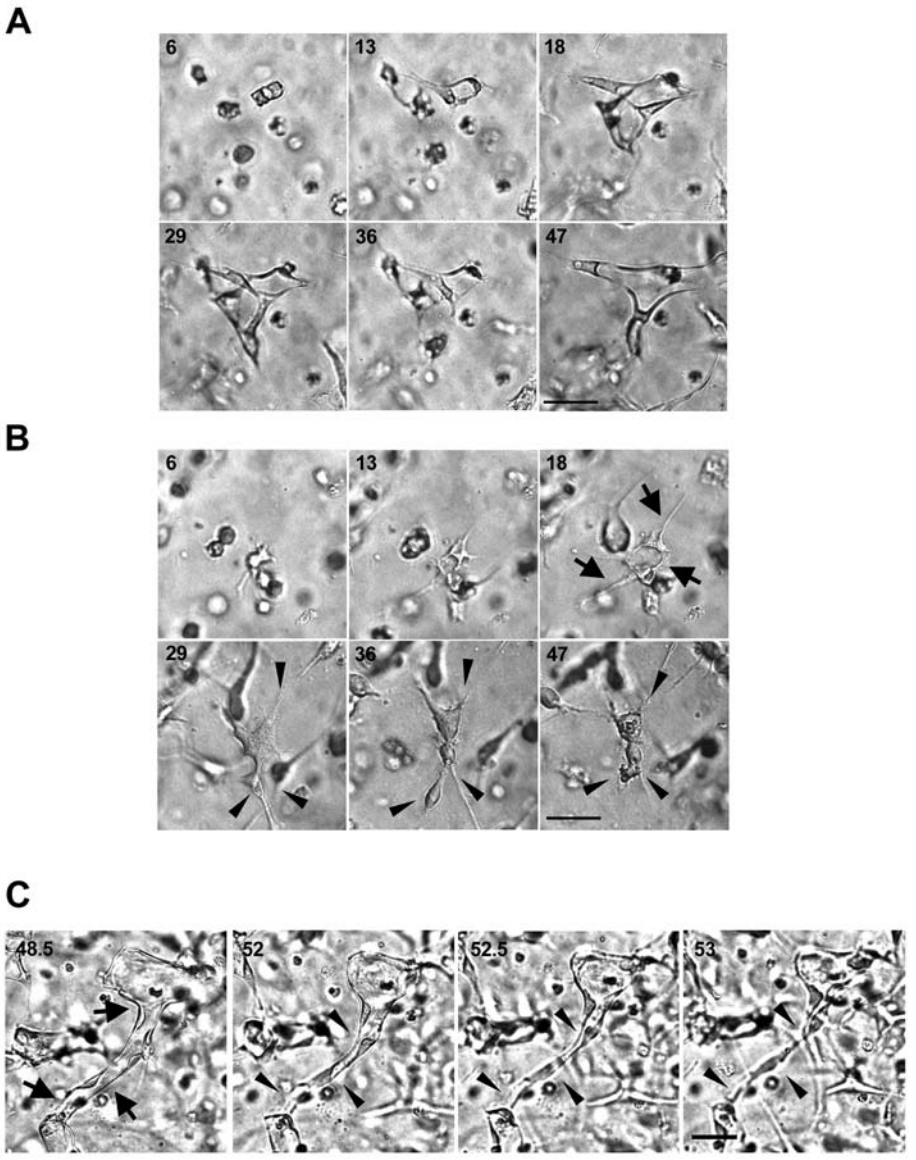

Fig. 2. Time-lapse photography of plasma kallikrein-induced MMP-1 activation and capillary tube regression. ECs were suspended in collagen matrices and cultured in the absence or presence of 1 μg/ml kallikrein. Sequential time-lapse photographs were taken of individual EC fields at the time in hours denoted in each panel. (A) In the absence of kallikrein, ECs form sprouts, vacuoles and tubular networks, a process known as morphogenesis. (B) In the presence of kallikrein, morphogenesis is initiated (arrows); however, capillary tubes and vacuoles collapse (arrowheads), eventually resulting in individual EC rounding, aggregation and death. (C) ECs were allowed to undergo morphogenesis and establish EC networks for 48 hours prior to the addition of kallikrein. An EC-lined tube (arrows) is visible undergoing tube regression (arrowheads) prior to the onset of gel contraction. Bar, 100 μm.

dependent tube regression is inhibited by both serine protease and MMP inhibitors.

Addition of plasma prekallikrein induces capillary tube regression only in the presence of Factor XII or HMW kininogen

It is well known that the EC cell surface is an important regulator of plasma prekallikrein activation via two pathways, Factor XII or high molecular weight kininogen (HMWK) (Colman, 1999; Kaplan et al., 2001; Mahdi et al., 2002; Schmaier, 2000; Schousboe, 2001). Dose-response experiments were performed with Factor XII zymogen, which was added to ECs in the 384 micro-well assay in the presence or absence of prekallikrein. As shown in Fig. 3B, the addition of Factor XII at doses as low as 32 ng/ml after 50 hours of culture induced capillary tube regression and collagen gel contraction only in the presence of plasma prekallikrein at 2 μg/ml. Western blot analysis of MMP-1 activation revealed that both Factor XII and prekallikrein were required to induce MMP-1 activation, which correlated with the tube regression and gel contraction response (Fig. 3B). The addition of Factor XII alone did not induce regression.

A dose-response experiment with varying doses of added HMWK was performed with 2 μg/ml prekallikrein in the presence of Zn^{2+}, Mn^{2+} or no added divalent cations (Fig. 3C). Previous work has indicated that Zn^{2+} ions enhance the ability of HMWK to bind to the EC cell surface and activate plasma prekallikrein (Joseph et al., 1996). Here, in the presence of 10 μg/ml HMW kininogen, collagen gel contraction occurred in the presence of prekallikrein without added divalent cations. However, at lower doses, the presence of Zn^{2+} ions but not Mn^{2+} markedly stimulated the ability of HMWK to induce collagen gel contraction. Doses of HMW kininogen at 1 μg/ml were sufficient to induce the regression response in the presence of both prekallikrein and Zn^{2+} ions. Zn^{2+} ions in the presence of HMWK and prekallikrein markedly induced MMP-1 activation, which directly correlated with capillary tube regression and collagen gel contraction. Overall, these data indicate that known physiological mechanisms to activate plasma prekallikrein on the EC surface lead to MMP-1 activation and capillary tube regression.

Activation of MMP-1 correlates with increased EC-derived collagenase activity during plasma kallikrein- and plasmin-induced capillary tube regression

Although activation of MMP-1 via western blot analysis correlated in each case with tube regression and gel contraction, we assayed conditioned media for total

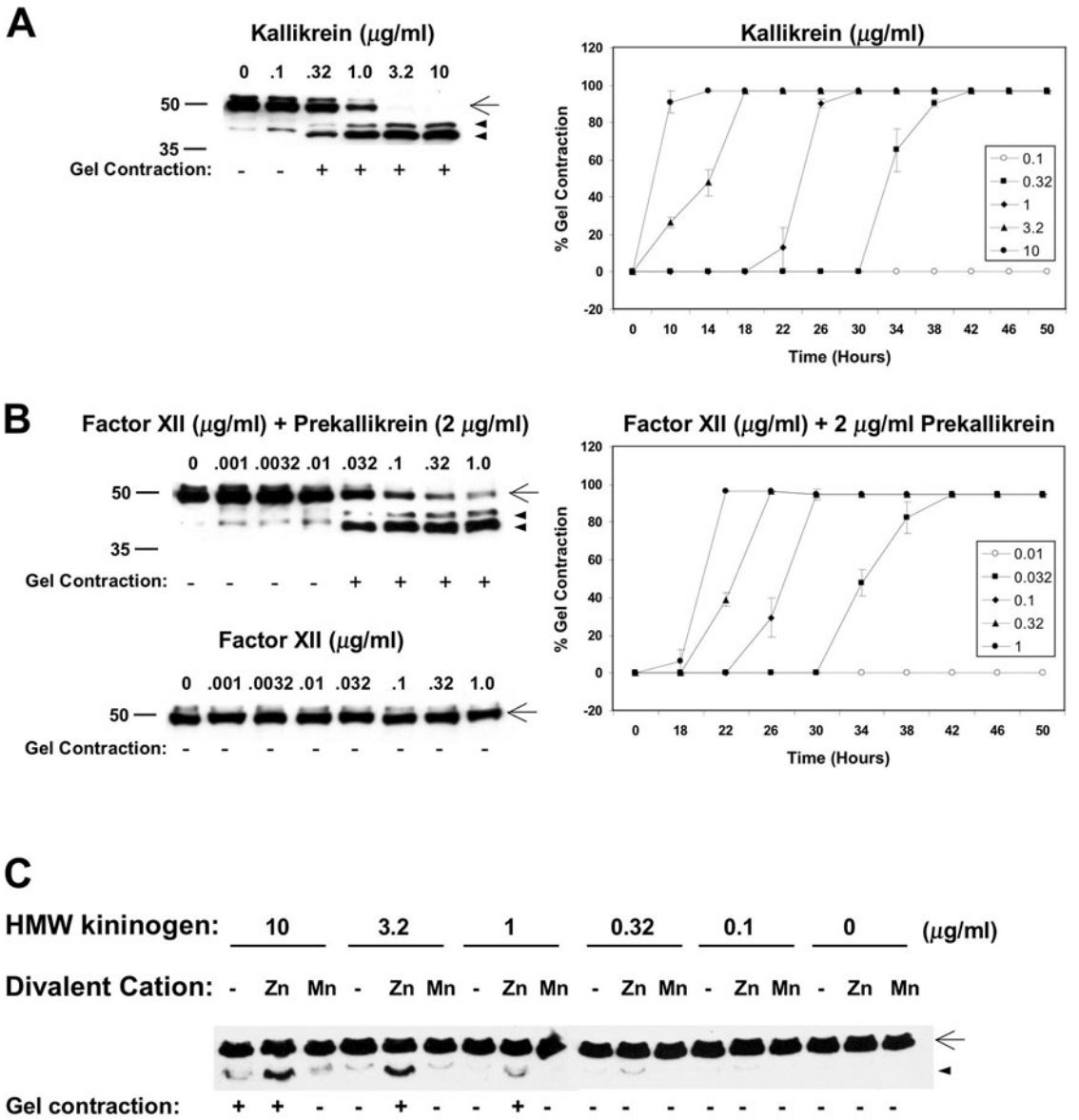

Fig. 3. Quantification of plasma kallikrein- or prekallikrein-mediated MMP-1 activation and capillary tube regression in 3D collagen matrices. ECs were suspended in collagen matrices and placed in a quantifiable 384 micro-well regression assay (15 μl gels, n=8 gels/condition) as shown in Fig. 1B. At time zero, media containing varying doses of active plasma kallikrein (A) or 2 μg/ml plasma prekallikrein and varying doses of Factor XII (B) were added to cultures. Collagen gels were monitored for tube regression and gel contraction over time and percentage of gel contraction was calculated (see Materials and Methods for details). Conditioned media were collected at 50 hours and analyzed for MMP-1 expression and activation. (C) ECs were suspended in collagen matrices. Culture media contained prekallikrein at 2 μg/ml, along with varying concentrations of HMW kininogen as indicated and either no cations or 100 μM $ZnCl_2$ or $MnCl_2$. Triplicate cultures were examined after 24 hours of culture for evidence of capillary tube regression and gel contraction (indicated by +) and analyzed for MMP-1. For all western blot analyses, arrows indicate the position of MMP-1 zymogen and arrowheads indicate activated MMP-1.

collagenase activity in a time-course experiment. ECs were cultured as previously described in the absence or presence of active plasmin or plasma kallikrein at two doses (0.5 μg/ml and 2.5 μg/ml). Conditioned media were collected at the indicated time points and were analyzed by western blotting and for collagenase activity using DQ collagen, a collagen preparation that emits limited fluorescence in its native, triple helical form, while emitting a strong fluorescent signal following proteolysis of the collagen triple helix (Della et al., 1999). Conditioned media from ECs cultured in the absence of plasmin or kallikrein (Control) showed minimal collagenase activity (Fig. 4) and western blot analysis of this media indicated that MMP-1 remained in its zymogen form (arrow). In contrast, the addition of either active plasmin or plasma kallikrein induced a marked dose- and time-dependent increase in collagenase activity that directly correlated with both the degree of MMP-1 activation visible on western blots (arrowheads) and time to 50% collagen gel contraction. The capillary tube regression and gel contraction response was determined as in Fig. 3 and the time to 50% gel contraction is shown above each data set.

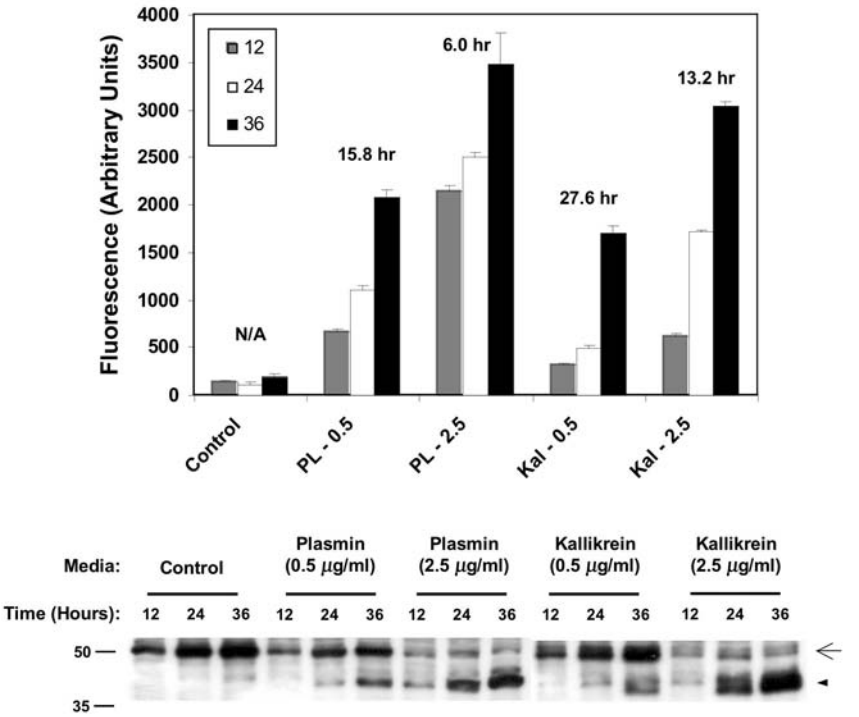

Fig. 4. Activation of MMP-1 correlates with increased collagenase activity in ECs during plasma kallikrein- and plasmin-induced capillary tube regression. ECs were suspended in collagen matrices and cultured in the absence (Control) or presence of active plasmin (PL) or kallikrein (Kal) at two doses (0.5 μg/ml, 2.5 μg/ml). Gels were monitored for tube regression and gel contraction via the 384 micro-well regression assay (as in Fig. 3). At the indicated time points, conditioned media were collected and MMP-1 levels were analyzed by western blotting. In addition, conditioned media were analyzed for collagenase activity via the DQ collagenase assay. Triplicate wells of conditioned media from the above time points were incubated with 25 μg/ml of DQ collagen overnight at room temperature. Absorbance was measured at 528±20 nm via a fluorescent microplate reader. Background fluorescence was subtracted prior to reporting of fluorescence in arbitrary units as mean±s.d. (n=3). Time to 50% gel contraction is reported above each data set. In each case, fluorescence corresponded with the degree of MMP-1 activation on the western blot and the time to 50% collagen gel contraction. Arrows and arrowheads indicate the position of MMP-1 in latent or activated forms, respectively.

Thus, the addition of both plasmin and kallikrein induced a dose-dependent activation of MMP-1, which led to time-dependent gel contraction and a corresponding increase in collagenase activity.

Multiple serine proteases induce MMP-1 zymogen activation, tube regression and gel contraction

The abilities of plasmin and plasma kallikrein to activate MMP-1 zymogen have been well described (Armour et al., 1984; Nagase et al., 1982; Nagase et al., 1990; Suzuki et al., 1990). Several other serine proteases (trypsin, neutrophil elastase, cathepsin G, tryptase and chymase) have been shown to active MMP zymogens in vitro (Duncan et al., 1998; Fang et al., 1997; Gruber et al., 1989; Okada et al., 1987; Sepper et al., 1997; Shamamian et al., 2001; Zhu et al., 2001); however, the ability of these serine proteases to activate MMP-1 zymogen in the context of tissue regression events is unclear at present. To address this question, dose-response experiments were performed using the purified serine proteases described above in the 384 micro-well regression assay. Addition of each serine protease resulted in a dose-dependent activation of MMP-1 (data not shown), which directly correlated with the time to initiation and completion of the tube regression and gel contraction response (Table 1). This in vitro data provides evidence that multiple serine proteases, present during physiologic and pathological vascular regression events in vivo, are capable of activating MMP-1 and inducing capillary tube regression and collagen gel contraction in vitro.

Inhibition of capillary tube regression using an MMP-1 siRNA

In order to directly demonstrate a requirement for MMP-1 (the downstream effector of these serine proteases and the collagenase responsible for collagen proteolysis) during human capillary tube regression and collagen gel contraction, ECs were transfected with MMP-1 siRNA or control siRNAs (luciferase, α2 macroglobulin). After transfection, ECs were resuspended in collagen gels in the 384 micro-well regression assay as described above. Upon completion of gel contraction, media were collected and western blot analyses were performed (Fig. 5). To evaluate the effect of decreased MMP-1 protein expression on the capillary tube regression and gel contraction response, four different serine protease conditions were added to culture media (active kallikrein, prekallikrein and Factor XII, neutrophil elastase, cathepsin G). Transfection of ECs with MMP-1 siRNA delayed the time to both onset and completion of gel contraction when compared to control siRNAs when media was supplemented with active kallikrein (0.5 μg/ml), or prekallikrein (2 μg/ml) and Factor XII (1 μg/ml) (Fig. 5A). Western blot analysis of culture media showed a marked decrease in MMP-1 protein levels compared to control cultures in all groups. Similarly, transfection of ECs with MMP-1 siRNA dramatically

Table 1. Multiple serine proteases are capable of activating MMP-1, leading to capillary tube regression and collagen gel contraction

Serine protease	Lowest dose (μg/ml) inducing tube regression	Time to 50% tube regression (hours)		
		Low dose*	Intermediate dose	High dose
Trypsin	0.1	34	10	3.5
Neutrophil elastase	0.5	49	25.5	23
Cathepsin G	0.5	37	27	11
Tryptase	0.5	45	24.5	17.5
Chymase	0.5	47.5	29	22

*Low, intermediate and high doses of enzyme were (in μg/ml): 0.1, 0.5 and 2.5 for trypsin; 0.5, 1.0 and 2.5 for neutrophil elastase, cathepsin G, tryptase and chymase.

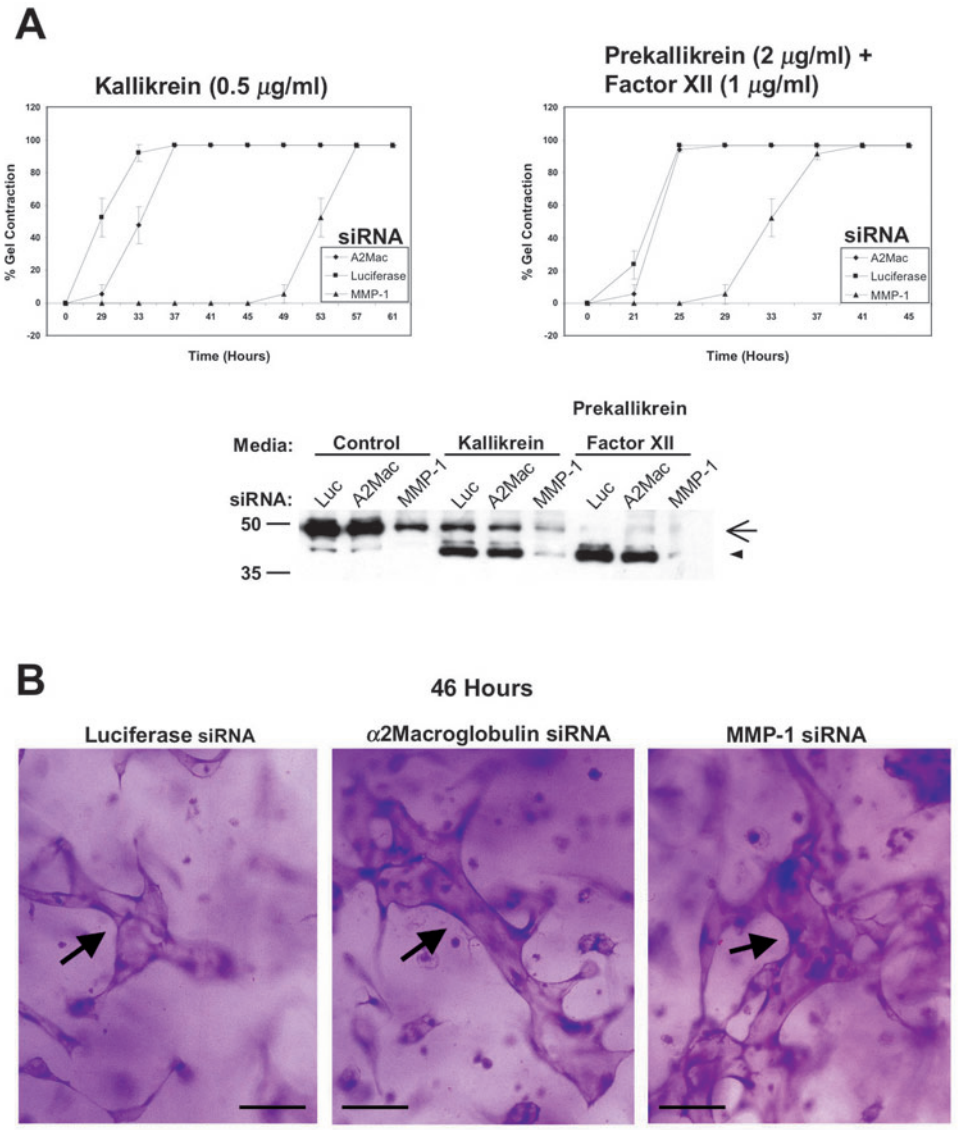

Fig. 5. Treatment of ECs with MMP-1 siRNA decreases MMP-1 protein expression and delays capillary tube regression and collagen gel contraction. ECs were transfected as described with MMP-1, α2 macroglobulin (A2Mac), or luciferase control siRNA duplexes at a final concentration of 200 nM, suspended in collagen matrices and placed in the quantitative 384 micro-well regression assay (as in Fig. 3). At time zero, culture media was added with or without serine proteases at the doses indicated and the percentage of gel contraction was calculated over time. (A) Culture media contained control media, plasma kallikrein, or prekallikrein and Factor XII at the indicated doses. Upon completion of gel contraction, conditioned media were collected and analyzed for MMP-1 expression and activation. (B) ECs transfected with the indicated siRNAs were established in collagen matrices for 46 hours in the absence of serine proteases and were fixed, stained and photographed. Arrows denote newly established EC vascular networks and open lumenal structures. Bar, 100 μm.

delayed the time to gel contraction when compared to control siRNAs in the presence of the neutrophil serine proteases, neutrophil elastase and cathepsin G (data not shown). Again, western blot analysis of culture media showed a similar decrease in MMP-1 protein levels in the presence of MMP-1 siRNA compared to luciferase and α2 macroglobulin (data not shown). Importantly, treatment of ECs with MMP-1 siRNA had no effect on the ability of ECs to undergo morphogenesis unlike control siRNA (Fig. 5B).

MMP-10 zymogen is induced during EC morphogenesis and is activated by multiple serine proteases

In order to investigate whether additional MMPs are involved in the capillary tube regression response, a time course experiment was performed with ECs during capillary tube morphogenesis in collagen matrices as previously described (Bell et al., 2001; Davis and Camarillo, 1996). Under serine protease-free conditions, conditioned media were collected at different time points and western blot analyses were performed to determine the presence of MMP-3, MMP-10 or MMP-13 (Fig. 6A). Although MMP-3 and MMP-13 were not detectable, MMP-10 zymogen was markedly induced and appeared to remain in its pro-enzyme form during capillary tube morphogenesis (Fig. 6A). To determine the ability of various serine proteases to activate MMP-10, dose-response experiments were performed utilizing ECs suspended in collagen matrices using the 384 micro-well assay. MMP-10 zymogen was activated in a dose-dependent manner by plasma kallikrein (Fig. 6B). Activation of MMP-10 by kallikrein directly corresponded with capillary tube regression and gel contraction (indicated by +). MMP-10 was also activated in a dose-dependent manner by trypsin, neutrophil elastase, cathepsin G, tryptase and chymase (data not shown). These data suggest that MMP-10 may represent a novel mediator of the capillary tube regression response.

An MMP-10 siRNA inhibits capillary tube regression

To determine a role for MMP-10 in capillary tube regression, ECs were transfected with an MMP-10 siRNA as described above. In these experiments, control siRNAs targeting luciferase (negative control) and MMP-1 (positive control) were used in addition to siRNAs targeting MMP-2 and MMP-9 to further evaluate the role of these MMPs. After transfection, ECs were resuspended in collagen gels in the 384 micro-well regression assay in the presence or absence of prekallikrein and Factor XII. Western blot analyses of MMP-10 and MMP-1 levels in conditioned media showed a marked decrease in MMP-10 and MMP-1 protein levels following

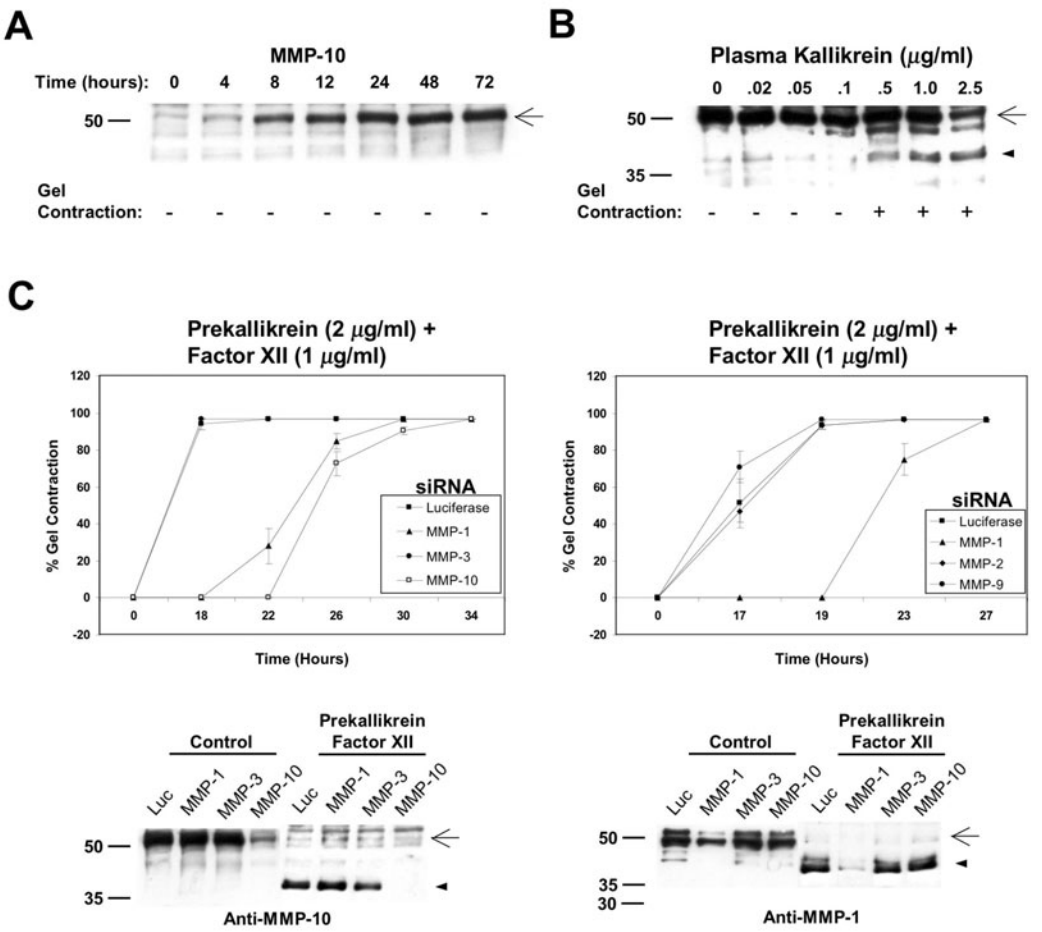

Fig. 6. MMP-10 is induced during EC morphogenesis, is activated by serine proteases and treatment of ECs with siRNA targeting MMP-10 delays capillary tube regression and collagen gel contraction induced by multiple serine proteases. ECs were suspended in collagen matrices as described in the absence (A) or presence (B) of varying doses of plasma kallikrein. Gels were monitored for contraction (indicated by +) and conditioned media were collected for western blot analysis of MMP-10 at the indicated time points (A) or at 72 hours (B). Note that latent MMP-10 (arrows) is visible in each condition; however, active MMP-10 (arrowheads) is visible only in the presence of kallikrein. (C) ECs were transfected as described with MMP-1, MMP-10, MMP-2, MMP-9 or luciferase control siRNA and suspended in collagen matrices. At time zero, culture media was added with or without prekallikrein (2 μg/ml) and Factor XII (1 μg/ml) and percentage gel contraction was calculated over time. Conditioned media were collected and analyzed for MMP-10 or MMP-1 expression and activation, as well as for MMP-2 and MMP-9 (data not shown). Arrows and arrowheads indicate the latent or activated forms of MMPs, respectively.

siRNA treatment (Fig. 6C). Importantly, both MMP-10 and MMP-1 siRNAs acted with specificity on their respective targets. In the presence of prekallikrein and Factor XII, transfection of ECs with MMP-10 siRNA delayed the time to both onset and completion of capillary tube regression and gel contraction when compared to the control (luciferase) (Fig. 6C). Treatment of ECs with MMP-10 siRNA had no effect on the ability of ECs to undergo morphogenesis compared to the control (data not shown), indicating that like MMP-1, MMP-10 is not involved in the formation of EC networks in 3D collagen matrices. Similar results were observed when ECs treated with MMP-1 or MMP-10 siRNAs were cultured in the presence of active kallikrein, neutrophil elastase, cathepsin G and plasminogen (data not shown). The capillary tube regression response was not delayed in ECs treated with MMP-2 or MMP-9 siRNA in the presence or absence of prekallikrein and Factor XII (Fig. 6C). Overall, these data provide strong evidence to support a major role for both MMP-1 and MMP-10 in mediating capillary tube regression and collagen gel contraction.

Increased expression of MMP-10 in ECs induces capillary tube regression and collagen gel contraction via activation of MMP-1 zymogen

To further define the involvement of MMP-10 (stromelysin-2) during capillary tube regression, a recombinant adenovirus encoding the gene for MMP-10 zymogen was generated. ECs were infected with control virus (GFP Ad) or MMP-10 virus (MMP-10 Ad) 24 hours prior to initiating cultures in the absence or presence of the serine proteases kallikrein, prekallikrein and Factor XII or plasminogen. Cultures were monitored over time for capillary tube regression and gel contraction and conditioned media were collected at the completion of gel contraction for western blot analysis of MMP-10. ECs infected with MMP-10 adenovirus induced a rapid capillary tube regression response (Fig. 7A), whereas ECs infected with control virus (GFP Ad) did not undergo regression in the absence of serine proteases (data not shown). Importantly, although each serine protease added to culture media accelerated regression and gel contraction when compared to control media, ECs expressing increased levels of MMP-10 were capable of initiating a regression response in the absence of any added serine protease. Two possible serine protease-independent mechanisms of MMP-10 activation include exposure to free radicals or auto-activation (Nakamura et al., 1998; Peppin and Weiss, 1986; Tyagi et al., 1993; Tyagi et al., 1996; Weiss et al., 1985). Confirming this result, western blot analysis of MMP-10 levels (Fig. 7A) showed minimal MMP-10 zymogen levels for ECs infected with GFP Ad, whereas ECs infected with MMP-10 Ad showed markedly

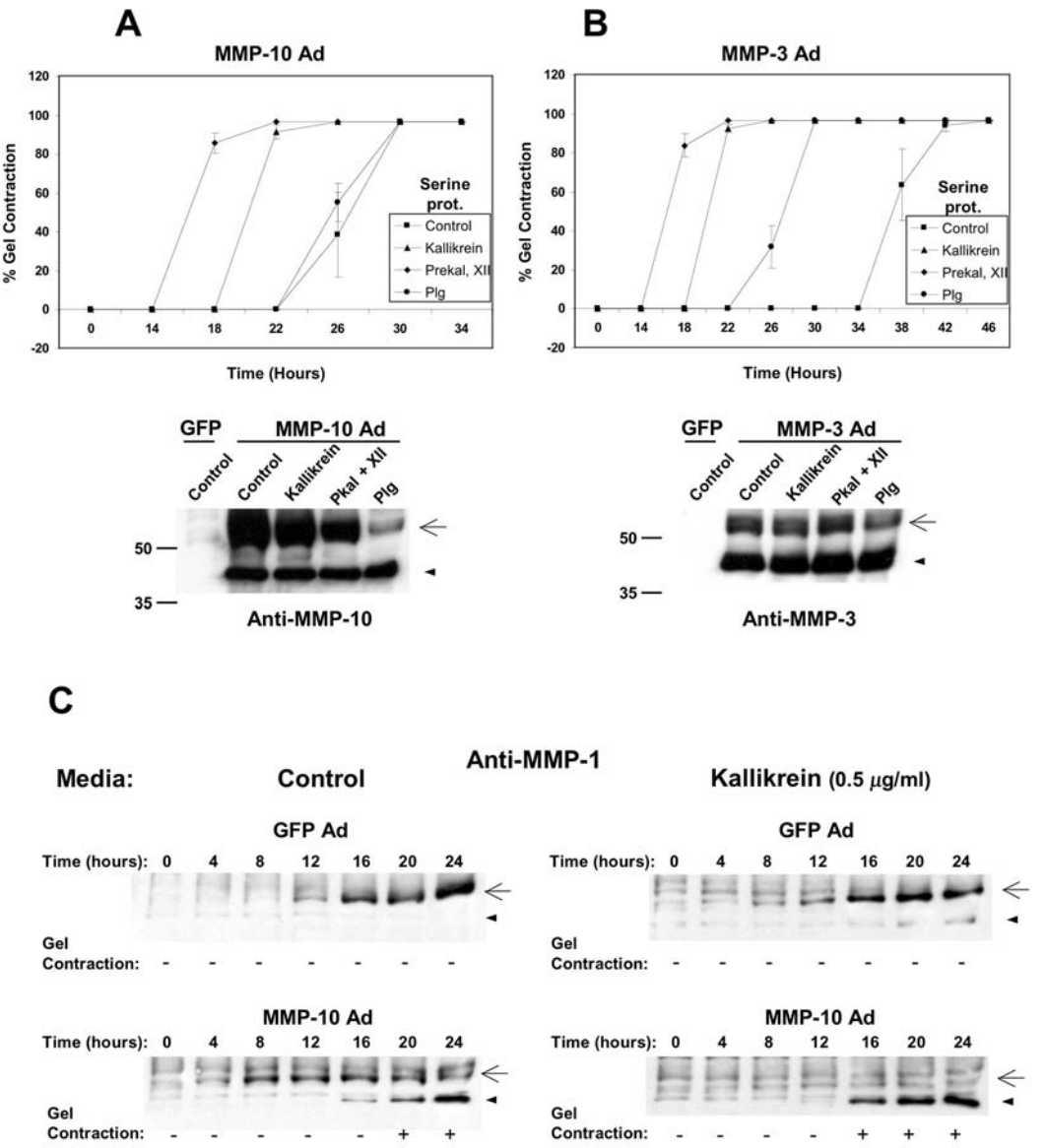

Fig. 7. Increased expression of stromelysin-2 (MMP-10) and stromelysin-1 (MMP-3) induce rapid capillary tube regression and collagen gel contraction via activation of MMP-1 zymogen. ECs were infected with GFP, MMP-10, or MMP-3 adenoviruses 24 hours prior to suspension in collagen gels. Gels were monitored over time for contraction and conditioned media were collected at the completion of the contraction response. (A) ECs infected with GFP Ad or MMP-10 Ad were cultured in control media or media containing active kallikrein (0.5 μg/ml), prekallikrein (2 μg/ml) and Factor XII (1 μg/ml) (Prekal, XII), or plasminogen (2 μg/ml) (Plg). Note that low levels of MMP-10 were present in GFP infected cells, whereas cells infected with MMP-10 Ad had increased levels of latent (arrows) and active (arrowheads) forms of MMP-10, regardless of the absence or presence of serine proteases. Addition of serine proteases accelerated capillary tube regression and gel contraction. (B) ECs infected with GFP Ad or MMP-3 Ad were cultured in control media or media containing kallikrein, prekallikrein and Factor XII, or plasminogen at the concentrations listed in panel A. No detectable MMP-3 was present in GFP infected cells, while cells infected with MMP-3 Ad had increased levels of latent (arrows) and active (arrowheads) forms of MMP-3, regardless of the absence or presence of serine proteases. In all cases, the addition of serine proteases led to more rapid capillary tube regression and gel contraction. (C) ECs infected with GFP Ad or MMP-10 Ad were cultured in the absence or presence of 0.5 μg/ml kallikrein. Gels were monitored for contraction (indicated by +) and conditioned media were collected at the indicated time points. Under control conditions, latent MMP-1 (arrows) was present in both GFP and MMP-10 expressing cells, however, active MMP-1 (arrowheads) was present only in cells expressing increased levels of MMP-10. In the presence of kallikrein, a small amount of active MMP-1 was present in the GFP-expressing cells, however, the presence of both kallikrein and MMP-10 substantially increased the amount of active MMP-1, which directly correlated with the time to tube regression and gel contraction.

increased levels of both latent (arrows) and active (arrowheads) MMP-10 regardless of the presence or absence of the described serine protease.

To investigate these findings, we prepared another adenoviral vector expressing the related stromelysin, MMP-3 (stromelysin-1). Additional cultures were established using ECs infected with control virus (GFP Ad) or the adenovirus encoding MMP-3 zymogen (MMP-3 Ad) 24 hours prior to addition to the 384 micro-well regression assay (Fig. 7B). Gels were monitored for contraction and conditioned media collected as described for Fig. 7A. Similar to increased MMP-10 expression, ECs infected with MMP-3 Ad induced a rapid capillary tube regression response (Fig. 7B), whereas ECs infected with control virus (GFP Ad) did not undergo regression in the absence of serine proteases (data not shown). Although each serine protease added to culture media accelerated regression and gel contraction when compared to serine protease-free media, MMP-3 infected ECs also were induced to regress in the absence of any added serine protease in a manner similar to ECs expressing increased levels of MMP-10. Western blot analysis of conditioned media showed no detectable MMP-3 zymogen levels for ECs infected with GFP Ad, whereas ECs infected with MMP-3 Ad showed markedly increased levels of both latent (Fig. 7B, arrows) and

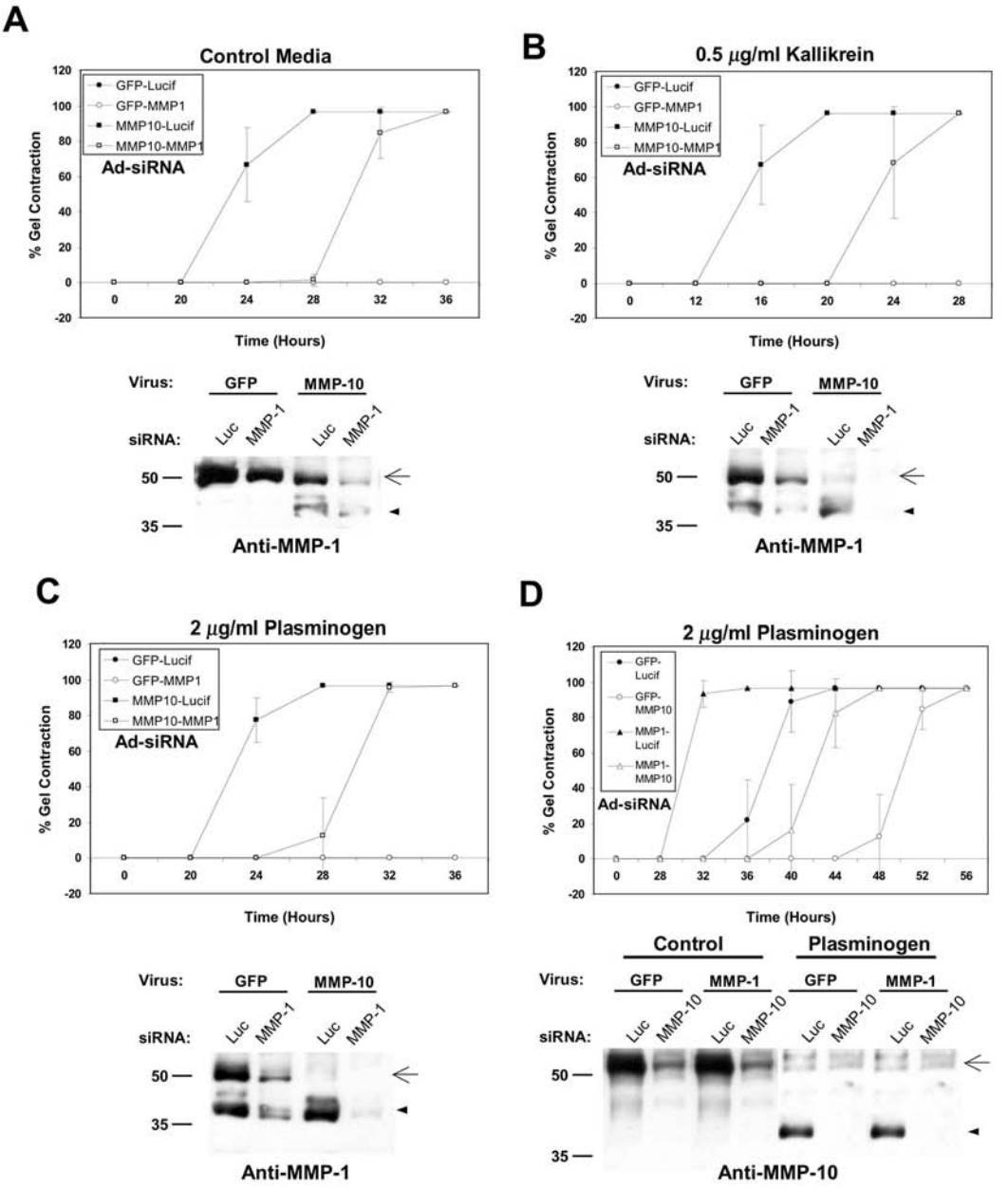

Fig. 8. MMP-1 and MMP-10 regulate EC tube regression in three dimensional collagen matrices. (A-C) ECs were transfected with siRNA against MMP-1 or luciferase control. Recombinant adenoviruses were used 24 hours later to deliver GFP or MMP-10. Cultures were established in the absence (A) or presence of 0.5 μg/ml kallikrein (B) or 2 μg/ml plasminogen (C). Gels were monitored every 4 hours for tube regression and percent gel contraction was recorded. At 56 hours, cultures were fixed and conditioned media were collected for western blot analysis of MMP-1 levels (A-C). Note that in all panels a decrease in latent MMP-1 was present in cells treated with MMP-1 siRNA compared to luciferase control. MMP-1 activation occurred due to an increase in MMP-10 levels and also in the presence of the serine proteases plasma kallikrein and plasminogen. (D) ECs were transfected with siRNA against MMP-10 or luciferase control as described. 24 hours later, ECs were infected with GFP-Ad or MMP-1 Ad. Cultures were established in the absence or presence of 2 μg/ml plasminogen. Cultures were monitored every 4 hours for tube regression and percentage gel contraction was recorded. At 56 hours, cultures were fixed and conditioned media were collected for western blot analysis of MMP-10 levels. Note that in all cases, a decrease in latent MMP-10 was present in cells treated with MMP-10 siRNA compared to controls. Arrows and arrowheads indicate the position of MMP-10 in latent or activated forms, respectively.

active (arrowheads) MMP-3 regardless of the presence or absence of the described serine protease. To elucidate the mechanism through which MMP-10 and MMP-3 activation occurs in the absence of added serine proteases, ECs were infected with GFP Ad, MMP-10 Ad, or MMP-3 Ad 24 hours prior to suspension in collagen gels as previously reported (Davis et al., 2001). Cultures were established in control media (without serine proteases) in the presence of: aprotinin (5800 KIU/ml), PAI-I (2.5 μg/ml), ecotin (25 μM), AEBSF (100 μM), TIMP-1 (3 μg/ml), α2 macroglobulin (25 μg/ml), or MMP Inhibitor-I (175 μM). Although regression was inhibited in the presence of TIMP-1, α2 macroglobulin and MMP Inhibitor I, regression was not sensitive to serine protease inhibitors (data not shown), suggesting that either ECs possess a mechanism to activate stromelysins in a manner that does not require serine proteases, or that these MMPs can auto-activate when expressed at sufficiently high levels.

Regulation of capillary tube regression by both MMP-1 and MMP-10

To determine if MMP-10 induces tube regression and gel contraction via activation of MMP-1, a time course experiment was performed utilizing ECs infected with either MMP-10 Ad or GFP Ad in the absence or presence of 0.5 μg/ml plasma kallikrein (Fig. 7C). Collagen gels were monitored for tube regression and gel contraction and conditioned media were collected at the indicated times. At 24 hours, cultures were

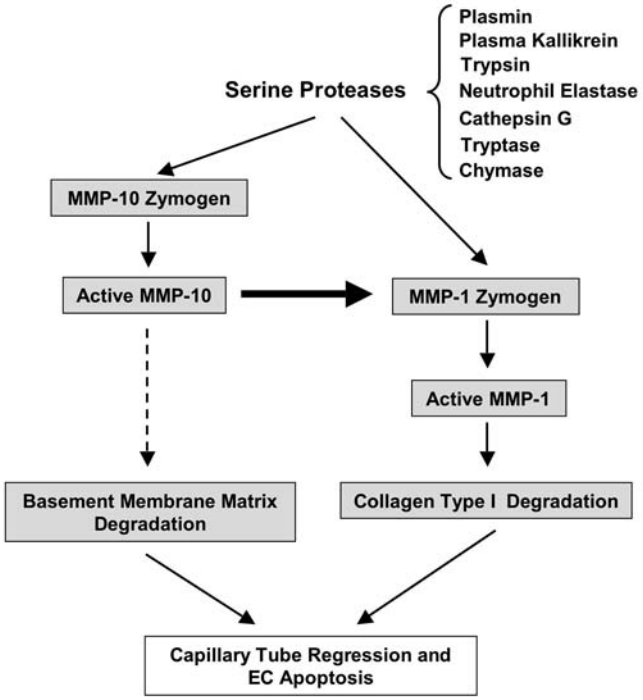

Fig. 9. Schematic diagram illustrating the ability of multiple serine proteases to activate MMP-1 and MMP-10 zymogens to control capillary tube regression in 3D collagen matrices. MMP-1 is activated directly by either serine proteases or activated MMP-10 (bold arrow). MMP-10 and serine proteases act synergistically to super-activate MMP-1, leading to type I collagen degradation, capillary tube regression and EC apoptosis. MMP-10 contributes to capillary tube regression by activating MMP-1 zymogen (bold arrows) and may contribute to degradation of basement membrane matrix leading also to tube regression and apoptosis (dashed arrow).

fixed and western blot analysis of MMP-1 activation was performed. Under serine protease-free conditions (control), ECs expressing GFP produced MMP-1 pro-enzyme (arrows). Active MMP-1 was detected only in ECs with increased MMP-10 expression (arrowheads). MMP-1 activation (arrowheads) correlated directly with the time of tube regression and gel contraction (indicated by +). In the presence of plasma kallikrein, activated MMP-1 was observed in ECs expressing GFP and MMP-10, and as expected, MMP-1 activation was enhanced (arrowheads) in ECs expressing increased levels of MMP-10 compared to cells expressing GFP. The presence of plasma kallikrein increased the amount of active MMP-1 and accelerated the time to gel contraction when compared to serine protease-free conditions. This experiment provides direct evidence that MMP-10 enhances activation of MMP-1, which directly correlates with tube regression and gel contraction.

To further evaluate the ability of MMP-10 to induce tube regression via activation of MMP-1, ECs were transfected with MMP-1 or luciferase control siRNA as described above. Twenty-four hours after the second transfection, ECs were infected with MMP-10 Ad or control virus (GFP Ad). Sixteen hours after adenoviral infection, ECs were suspended in collagen matrices in the 384 micro-well regression assay in the presence or absence (control) of the serine proteases plasma kallikrein or plasminogen. Addition of culture media denoted time zero. At 56 hours, conditioned media were collected for analysis of MMP-1 levels. ECs induced to express increased levels of MMP-10 and treated with control siRNA rapidly induced the tube regression and gel contraction response (Fig. 8A-C, closed boxes), however, ECs with increased expression of MMP-10 and treated with MMP-1 siRNA had a delayed tube regression response (open boxes). ECs infected with control virus (GFP Ad) did not undergo tube regression and gel contraction during the evaluated time course. The addition of plasma kallikrein (Fig. 8B) or plasminogen (Fig. 8C) to the culture medium accelerated the regression response in each case. Western blot analysis of MMP-1 revealed a marked decrease in total MMP-1 levels in ECs transfected with MMP-1 siRNA compared to the control. In the absence of serine proteases, MMP-1 remained latent in cells expressing GFP, but was activated by cells expressing increased levels of MMP-10 (Fig. 8A). Thus, MMP-1 siRNA markedly blocked the ability of increased MMP-10 expression to accelerate tube regression in ECs.

To provide further support for the dual role of MMP-1 and MMP-10 in tube regression, ECs were transfected with MMP-10 or luciferase control siRNA before being infected with adenoviruses encoding MMP-1 zymogen (MMP-1 Ad) or a control (GFP Ad) (Fig. 8D). ECs were suspended in collagen matrices in the 384 micro-well regression assay in the absence or presence of 2 μg/ml plasminogen. At 56 hours, conditioned media were collected for analysis of MMP-10 levels. ECs expressing increased levels of MMP-1 and treated with control siRNA rapidly induced the tube regression and gel contraction response at 32-36 hours (Fig. 8D, closed triangles), however, ECs expressing increased MMP-1 levels and treated with MMP-10 siRNA underwent delayed tube regression and gel contraction (open triangles, 40-48 hours). ECs infected with GFP Ad and treated with control siRNA induced tube regression at 36-44 hours (closed circles), whereas ECs infected with GFP Ad and treated with MMP-10 siRNA exhibited a delayed tube regression response (48-56 hours, open circles). Western blot analysis of MMP-10 indicated a marked decrease in MMP-10 levels for cells transfected with MMP-10 siRNA when compared to control siRNA. These experiments demonstrate that both EC-derived MMP-1 and MMP-10 are involved in regulating proteinase-induced capillary tube regression response in 3D collagen matrices.

Discussion

MMP-1, MMP-10 and multiple serine proteases regulate capillary tube regression events in three-dimensional collagen matrices

Prior reports have shown that proteinases regulate regression of capillary tubes in vitro (Davis et al., 2001; Davis et al., 2002; Zhu et al., 2000) and in vivo (Bajou et al., 1998; Bajou et al., 2001). In contrast, other work has shown that MT-MMPs (Knauper and Murphy, 1998) in particular, play an important role in stimulating the formation of capillary tubes in 3D extracellular matrices (Bayless and Davis, 2003; Davis et al., 2002; Hiraoka et al., 1998; Hotary et al., 2000). We have reported that ECs can induce capillary tube regression by generating the active enzyme plasmin from plasminogen, which further activates MMP-1 to induce collagen degradation

and capillary tube regression (Davis et al., 2001). Here, we report that multiple serine proteases induce activation of MMP-1 and MMP-10 to stimulate capillary tube regression. Plasma kallikrein, trypsin, neutrophil elastase, cathepsin G, tryptase and chymase have been reported to activate various MMP zymogens (Armour et al., 1984; Duncan et al., 1998; Fang et al., 1997; Gruber et al., 1989; Nagase et al., 1982; Okada et al., 1987; Sepper et al., 1997; Shamamian et al., 2001; Zhu et al., 2001). In this study, we report that multiple serine proteases are capable of activating EC-derived MMP-1 and MMP-10 zymogens in a dose-dependent manner. The combination of serine proteases and active MMP-10 leads to synergistic activation of MMP-1, which induces collagen proteolysis and subsequent breakdown of the matrix scaffold supporting capillary tube networks.

The concept that proteinases mediate capillary tube regression through ECM degradation is appealing for several reasons. It is well known that EC survival is dependent on EC-ECM adhesion, and disruption of these contacts results in EC apoptosis or anoikis (Frisch and Screaton, 2001; Meredith, Jr et al., 1993). Furthermore, the matrix scaffold is required for the integrity of EC-lined tubes in three dimensions (Davis et al., 2001; Davis et al., 2002; Vernon and Sage, 1995; Zhu et al., 2000) and physical alterations in this scaffold will cause tube collapse (Davis et al., 2001). Proteinase-mediated mechanisms underlie tissue regression phenomena in other tissues, such as the mammary gland. Werb and colleagues have shown in pioneering studies that proteinase-induced degradation of ECM controls mammary gland regression (Sternlicht and Werb, 2001; Sympson et al., 1994; Talhouk et al., 1992; Werb et al., 1996; Werb et al., 1999). Furthermore, plasma kallikrein, plasminogen and MMPs are involved in these events (Selvarajan et al., 2001; Werb et al., 1996), showing similar conclusions to the work presented here. Thus, it appears that proteinase-induced endothelial or epithelial tube regression is a phenomenon of general importance to tissue regression. In support of these conclusions, the proteinase inhibitor RECK knockout mouse resulted in a vascular lethal phenotype at embryonic day 10.5 (Oh et al., 2001). Analysis by electron microscopy and immunostaining showed a marked loss of collagen type I fibrils suggesting that the loss of RECK resulted in increased interstitial collagenase activity, which allowed collagen scaffold breakdown and vascular regression. These in vivo findings from the RECK knockout are remarkably similar to the in vitro model of collagen proteolysis and EC tube regression presented here. The nature of the mouse MMP that might represent the equivalent of human MMP-1 is not clear at present, but could include MMP-13, MMP-8, or others (Jeffrey, 1998). Interestingly, we have shown that adenoviral expression of murine MMP-13 in our EC culture system markedly accelerates the ability of serine proteases to induce EC tube regression (W.B.S. and G.E.D., unpublished observations). Thus, murine MMP-13 and human MMP-1 act as pro-regression agents, suggesting that murine MMP-13 may be capable of serving as the equivalent of human MMP-1 in regulating mouse capillary tube regression events.

Multiple serine proteases activate MMP-1 zymogen to regulate capillary tube regression in 3D collagen matrices

All of the serine proteases evaluated in this study caused MMP-1 activation that led to capillary tube regression. MMP-1, an interstitial collagenase, is activated in our system to initiate capillary tube collapse and collagen gel contraction. Previously, we reported that collagen type I degradation accompanied this regression response and that EC apoptosis also occurred during these events (Davis et al., 2001). It is also important to note that MMP-1, which is of central importance to the capillary tube regression phenomena presented here, has been reported to regulate uterine gland regression during the menstrual cycle (Curry and Osteen, 2001; Kokorine et al., 1996; Marbaix et al., 1996). MMP-1 has been shown to be transcriptionally regulated during this cycle and to be induced just prior to the initiation of gland regression. In this case, both endometrial and vascular tissue regression correlated with maximal MMP-1 expression. This work strongly implicates MMP-1 as a key factor in tissue regression phenomena in humans. In further support of such findings, a recent study showed that increased MMP-1 expression in human melanoma tumors correlated with tumor regression and a better clinical outcome (Nikkola et al., 2001). MMP-1 has also been reported to be a positive regulator of tumor invasion and keratinocyte migration (Benbow et al., 1999; Brinckerhoff et al., 2000; Dumin et al., 2001; Pilcher et al., 1997; Pilcher et al., 1998). Thus, MMP-1 and other MMPs appear to stimulate cell behaviors that are associated with tumor invasion and progression (Egeblad and Werb, 2002). Our previous work and the current study describe a new function for MMP-1, which is to regulate capillary tube regression in 3D ECM environments (Davis et al., 2001; Davis et al., 2002). The ability of various serine proteases to initiate MMP activation leading to collagen and ECM proteolysis and tissue regression is compelling. It appears that these pathways overlap and act synergistically to regulate ECM proteolysis. Although this report focuses on EC capillary tube regression, we believe the results are relevant to a wide variety of physiological and pathological conditions. In addition, the data presented in this study provide strong additional evidence to support a role for MMP-1 as a vascular regression factor and not as an EC pro-morphogenesis/invasion factor. Inhibition of MMP-1 expression levels via siRNA dramatically delayed capillary tube regression and gel contraction, while having no effect on ECs undergoing tubular morphogenesis (Fig. 5). During the later stages of wound healing, granulation tissue regresses and the ECM undergoes wound contraction (Clark, 1996). Consistent with the findings presented in this study, activation of MMP-1 during the later stages of wound healing might not only induce regression of granulation tissue, but also contribute to wound contraction. In support of this, several studies have detailed the ability of human fibroblasts and keratinocytes to contract collagen lattices in an MMP-1 or MMP-13 dependent manner, respectively (Netzel-Arnett et al., 2002; Pins et al., 2000).

MMP-10 (Stromelysin-2) is induced during EC morphogenesis and is activated by multiple serine proteases, leading to activation of MMP-1 and capillary tube regression

To our knowledge, this is the first report documenting the induction of MMP-10 protein during EC tubular morphogenesis in 3D matrices. The presence of a novel

stromelysin that remains latent in the absence of serine proteases, but is activated by multiple serine proteases is compelling for many reasons. Stromelysins are known to degrade components of the supporting ECM scaffold, namely basement membrane molecules such as collagen type IV, laminin and perlecan (Baricos et al., 1988; Bejarano et al., 1988; Nagase, 1995; Nagase, 1998). Additionally, it is known that MMP-10 is capable of activating other MMPs, specifically interstitial collagenases (MMP-1 and MMP-8), whereas MMP-3 has been shown to activate MMP-1, MMP-8 and MMP-13. Importantly, in the presence of serine proteases and stromelysins, MMP-1 is activated to a 'super-active' state with an approximately tenfold increase in its ability to degrade collagen (He et al., 1989; Suzuki et al., 1990). Thus, during EC tube regression and gel contraction, MMP-1 is not only activated directly by the serine proteases plasmin, kallikrein, neutrophil elastase, cathepsin G, tryptase and chymase, but is also activated directly by MMP-10 (Figs 3, 4, Fig. 7C, Table 1). The involvement of MMP-10 in the capillary tube regression response is shown in Fig. 6. siRNA targeting MMP-10 dramatically delayed tube regression and gel contraction in a manner similar to treatment with MMP-1 siRNA. Interestingly, in this experiment, treatment with MMP-2 or MMP-9 siRNA did not affect capillary tube regression. MMP-2 and MMP-9 are capable of degrading denatured collagen as well as other substrates; but importantly, serine proteases are also capable of degrading denatured collagens (Chung et al., 2004; Kapadia et al., 2004). The redundancy in enzymes that degrade denatured collagen may represent one reason why siRNA suppression of MMP-2 or MMP-9 expression has no effect on the tube regression response. Additional evidence supporting a key role of MMP-10 during tube regression is provided in Fig. 8. ECs expressing MMP-10 at increased levels underwent a capillary tube regression response in a manner that was dependent on MMP-1.

Thus, our data are consistent with the conclusion that EC-derived MMP-10 and serine proteases function cooperatively to activate MMP-1 in order to accomplish proteolysis of collagen type I, capillary tube regression and collagen gel contraction (Fig. 9). We present clear evidence that MMP-10 plays a role in MMP-1 activation in that increased expression of MMP-10 accelerates MMP-1 activation (Fig. 7C) and siRNA suppression of MMP-10 delays MMP-1-mediated tube regression and gel contraction (Fig. 6C, Fig. 8D). The influence of MMP-10 on MMP-1 activation directly correlates with capillary tube regression. Our data indicate that both MMP-1 and MMP-10 are involved in the capillary tube regression response in 3D collagen matrices that is stimulated by serine proteases. MMP-10 may also degrade basement membrane matrix components such as collagen type IV, laminin and perlecan, which are critical molecules in the assembly of EC-derived basement membranes. Importantly, stromal-derived MMP-3 is also a plausible candidate for contributing to vascular and other regression events in vivo because of its ability to degrade basement membrane matrix components and to synergistically activate MMP-1 with serine proteases. Further studies are necessary to examine the specific role of these enzymes in basement membrane degradation during capillary tube regression events. Thus, MMP-1 and MMP-10 are known to target the two key ECM components responsible for the establishment and maintenance of vascular tube networks, namely interstitial collagen type I matrix and the associated basement membrane matrix directly surrounding EC-lined tubes (Fig. 9) (Davis et al., 2002). Overall, these data further define critical molecular mechanisms involved in the disassembly of EC-lined tubes (i.e. capillary tube regression) and reveal a prominent role for specific MMPs (MMP-1 and MMP-10) as well as multiple serine proteases in these events.

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