Matrix Metalloproteinase-1 and Thrombin Differentially Activate Gene Expression in Endothelial Cells via PAR-1 and Promote Angiogenesis

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Many tumor types express matrix metalloproteinase-1 (MMP-1); its collagenase activity facilitates both tumor cell invasion and metastasis. MMP-1 expression is also associated with increased angiogenesis; however, the exact mechanism by which this occurs is not clear. MMP-1 proteolytically activates protease activated receptor-1 (PAR-1), a thrombin receptor that is highly expressed in endothelial cells. Thrombin is also present in the tumor microenvironment, and its activation of PAR-1 is pro-angiogenic. It is currently unknown whether MMP-1 activation of PAR-1 induces angiogenesis in a similar or different manner compared with thrombin. We sought to determine the mechanism by which MMP-1 promotes angiogenesis and to compare the effects of MMP-1 with those of thrombin. Our results demonstrate that via PAR-1, MMP-1 activates mitogen-activated protein kinase signaling cascades in microvessel endothelial cells. Although thrombin activation of PAR-1 also induces signaling through these pathways, the time-course of activation appears to vary. Gene expression analysis revealed a possible consequence of these signaling differences as MMP-1 and thrombin induce expression of different subsets of pro-angiogenic genes. Furthermore, the combination of thrombin and MMP-1 is more angiogenic than either protease alone. These data demonstrate that MMP-1 acts directly on endothelial cells as a pro-angiogenic signaling molecule and also suggest that the effects of MMP-1 may complement the activity of thrombin to better facilitate angiogenesis and promote tumor progression. (*Am J Pathol* 2008, 173:1736–1746; DOI: 10.2353/ajpath.2008.080512)

Matrix metalloproteinases (MMPs) are a closely related family of zinc-dependent proteolytic enzymes that function in the remodeling of the extracellular matrix and the proteolytic processing of bioactive molecules.¹ MMPs contribute to normal biological processes such as embryonic development and tissue repair, and also play a major role in the growth, invasion, and metastasis of malignant tumors.¹ In particular, tumor expression of the interstitial collagenase MMP-1 is associated with poor patient prognosis in malignant melanoma and in breast, ovarian, colorectal, pancreatic, and gastric cancers.² Many studies have linked the collagenase activity of MMP-1 to tumor cell invasion,^{2–4} and recently, we and others have found that MMP-1 expression is also associated with increased angiogenesis in xenograft models of melanoma,⁵ breast,^{6,7} and prostate⁸ tumors. Although the collagenolytic activity of MMP-1 may contribute to angiogenesis by clearing extracellular space to facilitate vessel branching,⁹ recent work has suggested that the activation of endothelial cell expressed protease activated receptor-1 (PAR-1) by tumor produced MMP-1 may also be pro-angiogenic.^{5,10} However, the mechanisms by which MMP-1 promotes angiogenesis through PAR-1 activation remain largely undefined.

PAR-1 is one of four proteolytically activated G-protein coupled receptors, which are expressed by a variety of cell types present in the tumor microenvironment, including endothelial cells, platelets, macrophages, and fibroblasts.¹⁰ The PARs are unique receptors in that they carry their own ligand, which is masked N-terminally under resting conditions. To activate the PAR, the masking peptide is cleaved by a protease; PAR-1, for example, is activated by MMP-1 and the serine proteases thrombin, factor Xa, and plasmin.¹¹ The exposed tethered ligand then binds to the extracellular active site on the PAR to

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activate it intramolecularly. Signal transduction initiated by PAR activation has many downstream consequences, leading to changes in cellular morphology, proliferation, migration, and adhesion.¹² Under normal conditions, PARs allow cells to respond to the proteolytically altered microenvironment present during development, wound healing, and inflammation.¹² The tumor microenvironment is very similar to that found during wound healing,¹³ and tumor cells may activate stromal PAR-1 by secreting proteases, such as MMP-1 and thrombin, to induce changes within the stromal cells and promote neoplastic progression.

Thrombin, the classic PAR-1 activator, is frequently present in the tumor microenvironment,^{14,15} and thrombin activation of PAR-1 expressed on endothelial cells has been consistently linked to angiogenesis, resulting in increased tumor growth and metastasis.^{14,16} MMP-1, secreted by both tumor and stromal cells, is also at high concentrations within the tumor microenvironment,⁵ and, like thrombin, MMP-1 is capable of cleaving PAR-1 to activate endothelial cells¹⁷; however, it is unknown if the effects of MMP-1 and thrombin on endothelial cells are redundant. MMP-1 and thrombin were recently shown to have different cleavage sites within the PAR-1 active site, suggesting that the two proteases may have distinct functions via PAR-1.¹⁸ Thrombin cleavage produces the classic PAR-1 activating hexapeptide, with the amino acid sequence S₄₂FLLRN₄₇. In comparison, the truncated ligand produced by MMP-1 cleavage, L₄₄RN₄₇, is predicted to have a low affinity for the PAR-1 active site¹⁸; the downstream effects of PAR-1 activation by MMP-1, and how they might differ from those of thrombin, are currently unknown.

Previously, we have found that tumor MMP-1 expression was strongly associated with angiogenesis in a melanoma xenograft model. The increase in vessel formation was correlated with tumor metastatic capability,⁵ suggesting that MMP-1-induced angiogenesis may be a critical aspect of neoplastic progression. Here, we define a potential mechanism by which MMP-1 may promote angiogenesis, using both purified MMP-1 and conditioned media from VMM12 melanoma cells, which contain MMP-1 as well as many other tumor secreted factors. In a defined system, we show that purified MMP-1 is sufficient to promote angiogenesis *in vivo*, and that these pro-angiogenic effects are significantly reduced when PAR-1 activation is inhibited. Further, MMP-1 was found to activate mitogen activated protein kinase (MAPK) signaling in endothelial cells via PAR-1, resulting in the induction of a panel of pro-angiogenic genes. These data demonstrate that, like thrombin, MMP-1 acts as a signaling molecule to elicit cellular responses. Additionally, direct comparisons to the effects of thrombin activation of PAR-1 revealed that the two proteases differentially induce gene expression in endothelial cells, and functional assays suggested that thrombin and MMP-1 may co-operate in the tumor microenvironment to better facilitate angiogenesis and promote tumor progression.

Materials and Methods

Reagents

Purified full-length MMP-1, supplied as a mixture of activated and pro-MMP-1, was purchased from Abcam (Cambridge, MA). Recombinant protein representing the MMP-1 catalytic domain was obtained from BioMol (Plymouth Meeting, PA). Purified human α -thrombin was from Hematological Technologies (Essex Junction, VT). The following antibodies were purchased from the indicated providers: anti-human MMP-1 and MMP-1 blocking antibody (Calbiochem, San Diego, CA), anti-FLAG (Sigma, St. Louis, MO), anti-phospho-map-erk-kinase (MEK) 1/2, anti-MEK1/2, anti-phospho-p38, and anti-p38 (Cell Signaling, Danvers, MA). The PAR-1 specific antagonist SCH79797 was obtained from Tocris (Ellisville, MO).

Cell Culture

Human microvessel endothelial cells (HMVECs) and VMM12 cells were cultured as previously described.^{5,19} Briefly, HMVECs were maintained on plates coated with Attachment Factor (Invitrogen, Portland, OR) in Medium 132 supplemented with Human Microvessel Growth Supplement (Invitrogen), and VMM12 cells were grown in 1× Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) at 37°C, 5% CO₂. In instances where serum-free media were used, Medium 132 was supplemented only with 0.2% lactalbumin hydrolysate (Hyclone).

Matrigel Plug Assay

The Matrigel plug assay was performed as previously described.²⁰ For these experiments, purified MMP-1 or α -thrombin was mixed with 500 μ l growth-factor reduced Matrigel (BD Biosciences, Sparks, MD) at 4°C to the concentrations indicated. An equal volume of 1× PBS was used as a control. For experiments using the PAR-1 inhibitor, Matrigel containing 5 nmol/L MMP-1 was mixed with either 200 nmol/L SCH79797 or an equal volume of dimethyl sulfoxide. The 500- μ l Matrigel mix was injected subcutaneously into the flank of female nude mice (strain nu/nu, Charles River), where it rapidly formed a plug. After 7 days, mice were sacrificed, and plugs were removed, fixed in 10% formaldehyde, and paraffin embedded. Four plugs were examined per group. Plugs were sectioned at 50 μ m intervals and three sections were stained with Masson's Trichrome to visualize infiltrating cells. Three additional sections from each plug were stained with anti-mouse CD31 (Abcam) to label endothelial cells, and microvessel density was quantified by counting the total number of positively stained vessels in each section, then normalized to the area of the plug within each section. Vessels within 0.3 mm of the plug in the surrounding stroma were also quantified. Only established vessels, >25 μ m in diameter, were counted; single CD31+ cells were not included. Micrographs were taken

using an Olympus IX50 inverted microscope equipped with a QImaging digital camera. All tissue staining was done by the Department of Research Pathology, Dartmouth Hitchcock Medical Center. Animal studies were approved by the Institutional Animal Care and Use Committee at Dartmouth College.

Cell Signaling Analysis

HMVECs (10^5) were plated in 6-well dishes in regular growth medium for 24 hours. Cells were washed once in PBS, and medium was switched to 1 ml serum-free medium for 2 hours. Purified α -thrombin or recombinant catalytic MMP-1 was added to the wells at the concentrations indicated, for the time indicated. For experiments using the MMP inhibitor, HMVECs were treated with either 5 μ mol/L MMP inhibitor II (Calbiochem), or an equal volume of DMSO. Cells were harvested and lysed using 2 $\times$ Laemmli buffer (Sigma). Western blot analysis was done as previously described,²¹ and blots shown are representative of at least three individual experiments. The integrated densities of each band from the phosphoblots were quantified using ImageJ software,²² and normalized to the integrated density from the corresponding bands of total protein.

PCR Array Analysis and Real-Time Reverse Transcription PCR

For PCR array analyses, 10^6 HMVECs were plated in 10 cm² dishes in regular growth medium for 24 hours. Cells were washed with 1 $\times$ PBS and media were switched to 4 ml Medium 132 containing only 1% FBS plus 5 nmol/L α -thrombin or 5 nmol/L full-length MMP-1. For experiments using the PAR-1 inhibitor, cells were pre-treated for 15' with 200 nmol/L SCH79797. After 24 hours, cells were harvested, and RNA extracted using the RNeasy RNA Isolation Kit (Qiagen, Valencia, CA). Reverse transcription (RT)-PCR was done using the RT² First Strand Kit (SuperArray, Frederick, MD) according to manufacturer's directions. Gene expression was measured by real-time PCR using the Human Angiogenesis RT² Profiler PCR array (SuperArray), following the manufacturer protocol. Data were analyzed using the $2^{\Delta\Delta C(t)}$ method.²³ For all other real-time RT-PCR analyses, RNA was reversed transcribed using TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA), and real-time PCR was done using the Sybr Green master mix (Applied Biosystems), both according to manufacturer's directions. We have previously listed the sequences of MMP primers⁵ and all other primers were purchased from Qiagen (Valencia, CA).

MMP-1 Immunoblot

HMVECs were plated at 10^5 cells/well, in 6-well plates. After 24 hours in regular growth media, cells were washed with 1 $\times$ PBS and 1 ml serum-free medium was added per well. HMVECs were treated with 5 nmol/L

purified, recombinant pro-MMP-1 (Calbiochem), or an equal volume of PBS for 24 hours. Proteins in the media were precipitated using 10% trichloroacetic acid and resuspended in 50 μ l 2 $\times$ Laemmli buffer, and 15 μ l were run on an SDS-polyacrylamide electrophoresis (PAGE) gel, with 10 ng pro-MMP-1 run in lane 1 as an unprocessed control. Immunoblots were done as described²¹ are representative of three separate experiments.

Tube Formation Assay

Wells of a 96-well plate were coated with 50 μ l growth-factor reduced Matrigel. After 30 minutes at 37°C to allow for Matrigel polymerization, 5×10^3 HMVECs were plated per well in Medium 132 containing 1% FBS. After 2 hours, media were aspirated, and replaced with either 100 μ l Medium 132 containing 1% FBS mixed with the indicated concentration of purified MMP-1 or α -thrombin, or 100 μ l of VMM12 conditioned media. For conditioned media, VMM12 cells were plated in regular growth media at 10^5 cells/well in 6-well dishes. After 24 hours, cells were washed with PBS and 1 ml of Medium 132 containing 1% FBS was added and conditioned for 24 hours. Media conditioned in a similar manner by HMVECs were used as a control. In instances where PAR-1 inhibitor was used, HMVECs were pretreated for 15 minutes with either 200 μ mol/L SCH79797 or an equal volume of DMSO before the addition of experimental treatments or conditioned media. For experiments using MMP-1 neutralizing antibody, either 1 μ g/ml MMP-1 blocking antibody or 1 μ g/ml anti-FLAG were added to conditioned media immediately before use in the experiment. After 24 hours, the cells were stained with CalceinAM vital dye (BD Biosciences), to better visualize tube formation, and micrographs were taken using an Olympus IX50 inverted microscope equipped with a QImaging digital camera. Images were converted to grayscale and colors were inverted using Adobe Photoshop. To measure tube area, the numbers of dark pixels in each image were quantified using Photoshop, for two images per well, with triplicate wells. Branch points were counted from three fields per well at $\times 20$ magnification, with triplicate wells. Data are representative of four individual experiments.

Statistical Analysis

Linear contrasts in one-way analysis of variance were used to calculate the statistical significance of all experiments, using JMP statistical software, version 5.01. Statistical significance was assigned to *P* values of <0.05 .

Results

MMP-1 is Pro-Angiogenic in Vivo

We have previously observed a strong correlation between MMP-1 expression and angiogenesis in a melanoma xenograft model.⁵ Using MMP-1 small hairpin RNAs in the VMM12 human melanoma cell line, we dem-

Figure 1. MMP-1 is pro-angiogenic *in vivo*. **A:** Matrigel was mixed with PBS (control), 5 nmol/L thrombin, or 5 nmol/L MMP-1, then injected subcutaneously into nude mice, as described in the Materials and Methods. After 7 days, Matrigel plugs and surrounding stromal tissue were removed and stained with Masson's Trichrome to visualize cells invading the Matrigel plug and anti-CD31 to highlight blood vessels within the plug and surrounding stroma. Micrographs are representative of sections from each group; **B:** Average number of CD31⁺ blood vessels per μm² plug, from **A**. **C:** Either 0.02% DMSO or 200 nmol/L of the PAR-1 inhibitor SCH79797 were mixed with Matrigel containing 5 nmol/L MMP-1, injected into nude mice, and analyzed as described above. **D:** Quantification of CD31⁺ vessels from **C**. Scale bars = 100 μm, **P* < 0.0005 compared to PBS, ***P* < 0.001 compared to 5 nmol/L MMP-1 plus DMSO, error bars are SD. **Arrows** highlight large vessels in the Matrigel plug or surrounding stroma.

onstrated that a 90% knock-down of MMP-1 expression by the tumor cells resulted in a significant decrease in both blood vessel number and size within the tumor microenvironment, as compared with control. Others have made analogous observations using breast^{6,7} and prostate tumor xenograft models,⁸ and together, these data suggest that MMP-1 may have an angiogenic function. MMPs, in general, are thought to promote angiogen-

esis by remodeling the extracellular matrix to allow for vessel branching, while simultaneously releasing growth factors embedded within the matrix.^{1,9,24} MMP-1 is also known to activate endothelial cell expressed PAR-1¹⁷; a similar thrombin-mediated activation of PAR-1 is known to be pro-angiogenic.^{14,16} However, a definitive link between MMP-1 and blood vessel formation has not yet been made.

Figure 2. MMP-1 induces activation of p38 and MEK/ERK signaling via PAR-1. **A:** Western blots of lysates from HMVECs treated for 5 minutes with either PBS (control) or 5 nmol/L MMP-1 in serum-free media. Cells in lanes 1 and 2 were pre-treated for 15 minutes with 0.02% DMSO (–), and cells in lane 3 were pre-treated with 200 μ mol/L SCH79797 (+). Proteins were resolved on a 10% SDS-PAGE gel and transferred to Immobilon membrane. Blots were probed with antibodies against phosphorylated p38 and phosphorylated-MEK1/2, then were stripped and re-probed with antibodies against total p38 and total MEK1/2. **B:** Western blots of lysates treated with either 5 nmol/L MMP-1 or 5 nmol/L thrombin for the times indicated. Blots were probed as described. **C:** Integrated densities of immunoblots shown in **B**. Phospho bands were normalized to the corresponding bands of total protein. * $P < 0.001$, MMP-1 treated compared to thrombin treated, ** $P < 0.001$, thrombin treated compared to MMP-1 treated. **D:** Immunoblots of lysates from HMVECs were pre-treated for 15 minutes with either 0.5% DMSO (–) or 5 μ mol/L MMP inhibitor II (+), then treated with MMP-1 for 15 minutes, as indicated. Immunoblots were performed. **E:** HMVECs were treated with either 0.5% DMSO (–) or MMP inhibitor (+) 15 minutes after the addition of 5 nmol/L MMP-1, as indicated.

Therefore, we used the Matrigel plug assay to first determine whether MMP-1 is sufficient to promote angiogenesis *in vivo*. This assay is a well-established system in which Matrigel is injected subcutaneously into mice, where it forms a solid plug that can support an extensive vascular response when the Matrigel is supplemented with angiogenic factors.^{20,25,26} Shown in Figure 1A, at 7 days postimplantation, the addition of 5 nmol/L purified MMP-1 or thrombin to the Matrigel resulted in a dramatic increase in the number of cells invading into the Matrigel from the surrounding stroma, as well as in the number of blood vessels either immediately surrounding the plug or within the plug itself. This is in contrast to PBS control plugs, which had very few invading cells or surrounding vasculature. Also, there was no significant difference between the numbers of CD31⁺ microvessels surrounding or within MMP-1 treated Matrigel plugs, compared to those containing thrombin (Figure 1, A and B), which were used as a positive control in these experiments. Thus, purified MMP-1 is pro-angiogenic, and MMP-1 and thrombin are equally able to induce angiogenesis *in vivo* in this system.

Additionally, the treatment of MMP-1-containing plugs with the specific PAR-1 antagonist SCH79797^{17,27,28} significantly ($P < 0.001$) reduced vessel formation, compared to MMP-1 plus DMSO control (Figure 1, C and D). These data suggest that PAR-1 activation may play an important role in the ability of MMP-1 to promote angiogenesis *in vivo*.

MMP-1 Induces MAPK Signaling in Endothelial Cells via PAR-1

PAR-1 is a G-protein coupled receptor; its activation can have many downstream consequences in endothelial cells that are ultimately pro-angiogenic.^{10,29} To define a mechanism by which MMP-1 may promote angiogenesis, several signaling cascades typically associated with PAR-1 activation were examined. Treatment of HMVECs with 5 nmol/L purified MMP-1, a dose previously shown to be sufficient for PAR-1 activation,^{7,17} induces phosphorylation of MEK1/2 and p38, components of two branches of the MAPK signaling cascade (Figure 2A). The other arm of this cascade, the JNK pathway, was not activated; neither were the nuclear factor (NF) κ B, phosphoinositol 3 kinase (PI3K), or Rho/ROK pathways (data not shown), which have previously been associated with the activation of PAR-1.^{30–32} Additionally, pretreatment of HMVECs with SCH79797 blocked the MMP-1 induced phosphorylation of MEK1/2 and p38 (Figure 2A), demonstrating that MMP-1 activation of PAR-1 induces MAPK signaling cascades in microvessel endothelial cells.

MMP-1 and Thrombin Differentially Induce MAPK Signaling

Recent work by Nesi and Fragai has shown that MMP-1 cleaves the PAR-1 exodomain at a different site than

Table 1. Pro-Angiogenic Gene Expression Differentially Regulated by MMP-1 and Thrombin

GenBank ID	Symbol	Description	Fold MMP-1 induction	Fold thrombin induction	Inhibited by SCH797997?
Induced only by MMP-1					
NM_002982	<i>CCL2</i>	Chemokine (C-C motif) ligand 2	2.87	ND	YES
NM_001565	<i>CXCL10</i>	Chemokine (C-X-C motif) ligand 10	3.01	ND	YES
NM_000800	<i>FGF1</i>	Fibroblast growth factor 1	2.08	ND	YES
NM_000584	<i>IL8</i>	Interleukin 8	2.81	ND	YES
NM_002210	<i>ITGAV</i>	Alpha V Integrin	2.45	ND	YES
NM_000212	<i>ITGB3</i>	Beta 3 Integrin	2.35	ND	YES
NM_000230	<i>LEP</i>	Leptin	2.21	ND	YES
NM_004557	<i>NOTCH4</i>	Notch homolog 4	2.81	ND	NO
NM_003376	<i>VEGFA</i>	Vascular endothelial growth factor A	3.34	ND	YES
Induced only by Thrombin					
NM_001146	<i>ANGPT1</i>	Angiopoietin 1	ND	4.08	NO
NM_014495	<i>ANGPTL3</i>	Angiopoietin-like 3	ND	36.76	YES
NM_002986	<i>CCL11</i>	Chemokine (C-C motif) ligand 11	ND	121.94	NO
NM_000618	<i>IGF1</i>	Insulin-like growth factor 1	ND	3.94	YES
NM_020405	<i>PLXDC1</i>	Plexin domain containing 1	ND	2.62	NO
NM_002615	<i>SERPINF1</i>	Serpin peptidase inhibitor clade F member 1	ND	2.46	NO
NM_000594	<i>TNF</i>	Tumor necrosis factor	ND	4.29	YES
Induced by both MMP-1 and Thrombin					
NM_005163	<i>AKT1</i>	V-akt murine thymoma viral oncogene homolog-1	2.39	6.23	NO
NM_001147	<i>ANGPT2</i>	Angiopoietin 2	3.07	3.76	NO
NM_001039667	<i>ANGPTL4</i>	Angiopoietin-like 4	104.69	129.79	YES
NM_007015	<i>LECT1</i>	Leukocyte cell derived chemotaxin 1	2.19	7.06	YES
NM_004994	<i>MMP9</i>	Matrix metalloproteinase 9	4.66	2.85	YES
NM_003238	<i>THBS1</i>	Thrombospondin 1	2.13	2.35	YES
NM_005429	<i>VEGFC</i>	Vascular endothelial growth factor C	8.69	5.43	NO

ND = no difference between control and treated groups.

thrombin, suggesting that MMP-1 and thrombin unmask different PAR-1 activating ligands.¹⁸ The ligand produced by MMP-1 cleavage is predicted to have a lower affinity for the PAR-1 active site than the thrombin produced ligand, but the functional consequences of this are unknown.¹⁸ Thrombin activation of MAPK signaling through PAR-1 is very rapid and transient, with maximal effects within 5 minutes.³³ We hypothesized that, if the MMP-1 produced ligand was indeed of lower affinity, the time course of MAPK activation between MMP-1 and thrombin would vary. As shown in Figure 2, B and C, phosphorylation of MEK1/2 and p38 occurred within 2 minutes of thrombin addition, and began to dissipate within 15 minutes. MMP-1 induction of MAPK signaling, however, did not occur until 5 minutes after the addition of MMP-1 to the HMVEC media, with a maximal activation at 15 minutes that continued for at least 60 minutes after treatment. Additionally, while pretreatment of endothelial cells with an MMP inhibitor blocked activation of the MAPK kinases (Figure 2D), the addition of the MMP inhibitor at 15 minutes after the initial MMP-1 treatment did not adversely affect signaling at the 30- and 60-minute time points (Figure 2E), suggesting that once MMP-1 has cleaved PAR-1 to activate signaling cascades, additional catalysis by MMP-1 is not necessary for signaling to continue. Together, these data demonstrate that the mechanisms by which MMP-1 and thrombin activate PAR-1 may be distinct, and suggest that the effects that the two proteases have on endothelial cells may not be redundant.

MMP-1 and Thrombin Induce Expression of Different Subsets of Pro-Angiogenic Genes

MAPK signaling induces the expression of a variety of genes, and microarray analyses have shown that thrombin induces several pro-angiogenic genes via PAR-1.^{34,35} To better define a mechanism by which MMP-1 may promote angiogenesis through PAR-1 activation, we used a PCR-based array kit to examine the expression of genes commonly induced during vessel formation, and then compared MMP-1 mediated changes to those of thrombin. Array results are listed in Table 1, with an induction of ≥ 2 fold considered noteworthy. Of the 80 genes examined, MMP-1 induced the expression of 16 pro-angiogenic genes, and, importantly, 9 genes were specific to MMP-1; thrombin could not induce their expression. Likewise, thrombin induced the expression of 14 genes in the array, 8 of which were specific to thrombin. Genes from each group in Table 1 were then randomly selected and mRNA expression in MMP-1 and thrombin treated HMVECs was examined by real time RT-PCR to validate the array (Figure 3A). Several of the MMP-1 specific genes have been associated with inflammation (CCL2 and interleukin [IL]8) and cell migration (CXCL10 and the α V and β 3 integrins),^{36–39} and several thrombin specific genes are known to act as growth factors (Angiopoietin-1, insulin-like growth factor [IGF]1, pigment epithelium-derived factor [SERPINF]1, and tumor necrosis factor [TNF]).^{40–42} Both MMP-1 and thrombin induced the expression of very potent pro-angiogenic

Figure 3. Validation of human angiogenesis PCR array. **A:** HMVECs were treated with PBS (control), 5 nmol/L MMP-1, or 5 nmol/L thrombin for 24 hours; mRNA expression of the indicated genes was then measured by real time RT-PCR. * $P < 0.02$ compared to control. **B:** HMVECs were pre-treated for 15 minutes with 0.02% DMSO or 200 nmol/L SCH79797, then treated with PBS (control), 5 nmol/L MMP-1, or 5 nmol/L thrombin for 24 hours. Expression by cells treated with DMSO were not significantly different from results shown in A. ** $P < 0.01$ compared to control. **C:** Gene expression induced in HMVECs by treatment with VMM12 conditioned media for 24 hours. Media conditioned by HMVECs were used as control. For some experiments, VMM12 conditioned media were first treated with 1 μ g/ml MMP-1 neutralizing antibody. Treatment with 1 μ g/ml anti-FLAG was not significantly different from cells treated with VMM12 conditioned media alone. Control wells were treated with media conditioned by HMVECs. *** $P < 0.001$, compared to cells treated with MMP-1 neutralized conditioned media. For all experiments, bars represent average fold induction, measured by $2^{\Delta\Delta C_T}$, compared to control, and are representative of three experiments. Error bars are SD.

genes, such as vascular endothelial growth factors (VEGFs), angiopoietins, and MMP-9.^{24,40,43}

To verify that MMP-1 and thrombin induced gene expression by activating PAR-1, the PCR array was repeated using RNA from HMVECs pre-treated with 200 nmol/L SCH79797 before protease addition. Although a majority of gene expression was blocked with the PAR-1 antagonist, a small number of the genes induced by MMP-1 and thrombin were not affected (Table 1 and Figure 3B). Induction of these genes was not significantly blocked with up to 500 nmol/L SCH79797 (data not shown); higher doses of SCH79797 could not be examined, as they were toxic to cells after 24 hours. Although thrombin may also signal through PAR-4 to induce gene expression,³² MMP-1 does not activate any other PAR.⁷

The mechanism by which MMP-1 induces the expression of Notch4, Akt1, and VEGFC is, therefore, unclear.

To demonstrate the biological relevance of tumor produced MMP-1 in inducing pro-angiogenic gene expression, we next treated HMVECs with media conditioned by VMM12 cells, and then examined the mRNA expression of several genes from Figure 3, A and B. Treatment of endothelial cells with melanoma conditioned media caused a strong induction in MMP-9, CCL2, and IL8 gene expression, which was significantly ($P < 0.0001$) reduced when a MMP-1 neutralizing antibody was added to the media (Figure 3C). It is important to note that expression was not completely returned to control levels when MMP-1 activity was blocked, indicating that other secreted factors in the media also effect expression of these genes. Additionally, VMM12 conditioned media, which does not contain thrombin (data not shown), could only slightly induce the expression of IGF-1, which the PCR array indicated was a thrombin specific gene. Together, these data serve to further validate the array results and demonstrate that MMP-1 is an important pro-angiogenic factor among the secreted milieu of a human melanoma cell line.

MMP-1 Induction of Other MMPs: Cross Talk between Tumor and Endothelial cells

The above data demonstrate that MMP-1 induces MMP-9 expression in HMVECs. To determine whether MMP-1 is inducing other MMPs not included in the angiogenesis array, we examined the MMP profile of HMVECs treated with VMM12 conditioned media. As shown in Figure 4A, of the MMPs commonly associated with neoplastic progression,⁴ only MMP-3 and MMP-9 were induced in the HMVECs by the conditioned media. Gene expression was significantly ($P < 0.001$) reduced when conditioned media were treated with MMP-1 neutralizing antibody, or when HMVECs were pre-treated with the PAR-1 inhibitor, suggesting that, in the VMM12 conditioned media, MMP-1 may be acting through PAR-1 to induce the expression of these other MMPs.

Additionally, MMP-3 is known to activate latent MMP-1 by cleaving the zymogen pro-domain to produce functional enzyme.⁴⁴ When HMVECs were incubated with pro-MMP-1 for 24 hours, the endothelial cells processed the protein into the 42kD and 22kD active forms^{45,46} (Figure 4B). This is suggestive of a feed-back mechanism between tumor and endothelial cells, where HMVECs initially process the pro-MMP-1 secreted by tumor cells into active enzyme, and the activated MMP-1 then induces higher levels of MMP-3 expression in the endothelial cells, allowing them to activate more pro-angiogenic MMP-1 within the microenvironment. This type of cross talk between tumor and stromal cells is vital for tumor progression,^{47,48} and suggests that endothelial cells have mechanisms in place to readily respond to tumor produced MMP-1 to promote angiogenesis.

MMP-1 and Thrombin Cooperate to Promote Tube Formation

Our data suggest that PAR-1 activation by MMP-1 and thrombin have different downstream consequences in

Figure 4. MMP-1 induces MMP-3 and MMP-9 expression. **A:** MMP profile of HMVECs treated with media conditioned by HMVECs (control), with media conditioned by VMM12 cells, with conditioned media containing 1 μ g/ml MMP-1 neutralizing antibody, or HMVECs were pre-treated for 15 minutes with 200 nmol/L SCH79797 before the addition of conditioned media. DMSO (0.02%) and 1 μ g/ml anti-FLAG had no significant effect on gene expression compared to VMM12 conditioned media alone. Bars represent the average fold induction compared to control for four experiments, error bars are SD, * $P < 0.001$, conditioned media compared to conditioned media with MMP-1 neutralizing antibody and HMVECs pre-treated with SCH79797. **B:** MMP-1 Western blot. **Left lane** is 10 ng purified pro-MMP-1, **center lane** shows MMP-1 expression by HMVECs, **right lane** shows activation of 5 nmol/L purified MMP-1 after 24 hours in culture with HMVECs. Pro-MMP-1 is typically 52 kD, and can be cleaved by MMP-3 to a 42-kD active form, a 22-kD active form, and a 27-kD inactive form. Blot is representative of three experiments.

both signal transduction and gene expression. To determine the functional effects of these differences, HMVECs were used in the tube formation assay, a well-established *in vitro* measure of angiogenesis.^{25,49} PAR-1 activation by thrombin has been previously reported to have bimodal effects in tumor cells, with lower thrombin concentrations acting as a mitogen, and higher concentrations inducing apoptosis.⁵⁰ We have found a similar bimodal effect of thrombin on endothelial cells, demonstrated in Figure 5,

A and B, where lower concentrations of thrombin (1–10 nmol/L) in the HMVEC media promote tube formation and branching morphogenesis, while higher doses (25 to 100 nmol/L) inhibit it. MMP-1, however, continues to induce tube formation at 100 nmol/L, a concentration of thrombin that was toxic to HMVECs. It is important to note that at very high doses (50 nmol/L and 100 nmol/L, Figure 5B), the amount of tube formation and branching morphogenesis induced by MMP-1 begins to decline, suggesting

Figure 5. MMP-1 and thrombin differentially induce endothelial cell branching *in vitro* in tube formation assays. **A:** HMVECs were plated on Matrigel and treated for 24 hours with the indicated doses of MMP-1 or thrombin in basal media containing 1% FBS. Control wells were treated with media alone. Micrographs are representative of four separate experiments. Scale bar = 50 μ m. **B:** Quantification of tube area per field and numbers of branch points per field from **A**. Three fields were examined per well, with three wells per dose, per experiment. The data are representative of four separate experiments. ** $P < 0.00001$, MMP-1 dose compared to equal thrombin dose, * $P < 0.001$, MMP-1 compared to control.

Figure 6. Thrombin enhances the tube formation and branching morphogenesis induced by VMM12 conditioned media, but thrombin cannot replace the pro-angiogenic effects of MMP-1 in tumor cell conditioned media. HMVECs were plated on Matrigel in tube formation assays and then treated with media conditioned for 24 hours by either VMM12 cells or HMVECs (control). Either PBS or 5 nmol/L thrombin was added to the media as indicated. For the some experiments, 1 μ g/ml MMP-1 neutralizing antibody was added to the conditioned media immediately before use in the assay, or HMVECs were pre-treated for 15 minutes with 200 nmol/L SCH79797. Neither 1 μ g/ml anti-FLAG nor 0.02% DMSO had any significant effect on tube formation or branching, compared to conditioned media alone. * P < 0.05, conditioned media plus thrombin compared to media with PBS. ** P < 0.001, conditioned media plus 5 nmol/L thrombin compared to media with PBS. *** P < 0.0001, conditioned media compared to media with MMP-1 neutralizing antibody. ** P < 0.001 media plus 5 nmol/L thrombin, compared to conditioned media with MMP-1 neutralizing antibody plus 5 nmol/L thrombin.

that, like thrombin, the pro-angiogenic effects of MMP-1 may be bimodal and dose-dependent.

The presence of thrombin in the tumor microenvironment has been well-documented⁵¹ and is associated with tumor progression in several types of cancers.⁵² To examine the combined effects of thrombin and MMP-1, which more closely mimics the tumor microenvironment, HMVECs were treated with media conditioned by VMM12 melanoma cells, with and without 5 nmol/L thrombin. The addition of thrombin induced significantly more tube formation (P < 0.05) and branching (P < 0.0001) than conditioned media alone (Figure 6). VMM12 conditioned media contains several secreted pro-angiogenic factors besides MMP-1, such as IL-8, VEGF, and fibroblast growth factor (data not shown); when MMP-1 activity in the conditioned media was blocked with a neutralizing antibody, tube formation and branching morphogenesis, therefore, were not returned to control levels. However, *in vitro* angiogenesis was significantly reduced (P <

0.0001) with the MMP-1 neutralizing antibody, indicating that MMP-1 is an important pro-angiogenic factor within the secreted milieu of the VMM12 cells. Importantly, when thrombin was added to VMM12 conditioned media treated with MMP-1 neutralizing antibody, tube formation and branching were not completely rescued, suggesting that thrombin alone cannot compensate for the effects of MMP-1. Treatment of HMVECs with the PAR-1 inhibitor reduced tube formation in wells treated with both conditioned media alone and media plus thrombin, suggesting that tube formation in this system is largely due PAR-1 activation. These data suggest that the effects of thrombin and MMP-1 within the tumor microenvironment may be additive, with the both proteases acting on endothelial cells and inducing angiogenesis to a greater extent than either could alone.

Discussion

It is increasingly recognized that the functions of MMPs extend far beyond their matrix degrading capabilities. MMPs are known to activate latent secreted signaling molecules, process receptor ligands, and cleave cell surface molecules and chemokines to affect cell adhesion and migration.^{1,53} This growing list of MMP capabilities demonstrates the importance of MMPs in both normal biological function and neoplastic progression. Recently, MMP-1 was shown to activate the G-protein coupled receptor PAR-1, which is expressed by many cell types within the tumor microenvironment. Although the effects of the MMP-1/PAR-1 signaling axis are still being defined, it is likely that MMP-1 will be shown to have an important role in tumor progression that extends beyond its collagenolytic activity. In this work, we show that, via PAR-1, MMP-1 induces MAPK signaling cascades (Figure 2A) and modifies gene expression (Table 1) in human microvessel endothelial cells to promote a pro-angiogenic program. These data more definitively establish MMP-1 as a signaling molecule that can elicit direct cellular effects to promote tumor progression, and further expands the repertoire of MMP functions.

Matrix remodeling is a critical aspect of tumor progression, and the type I collagenase activity of MMP-1 has long been associated with tumor growth, invasion, and metastasis.^{2-4,24} Angiogenesis is also a vital part of these processes,^{24,54} and our findings suggest that MMP-1 contributes to the dual functions of both modifying the matrix and promoting vessel formation. Indeed, several groups have observed a correlation between MMP-1 expression, angiogenesis, and tumor progression in melanoma, prostate, and breast cancer xenograft models.⁵⁻⁸ Our findings show that purified MMP-1 is capable of inducing angiogenesis *in vivo* in a controlled Matrigel system (Figure 1), thereby defining MMP-1 as a pro-angiogenic factor.

The pro-angiogenic effects of MMP-1 in the presence of thrombin are especially important to consider. Thrombin is a central regulator of vascular biology, and as such, is known to contribute to tumor progression and metastasis.^{11,14,55} Because both proteases activate PAR-1, the presence of both MMP-1 and thrombin in the tumor microenvironment may be redundant. However, our results

suggest that MMP-1 and thrombin have distinct effects on microvessel endothelial cells, with thrombin and MMP-1 differentially activating MAPK signaling (Figure 2) and gene expression (Table 1) via PAR-1. Interestingly, we have found that MMP-1 activation of MAPK signaling pathways is slower, but more durable, than thrombin induced MAPK signaling (Figure 2, B and C). MMP-1 has been shown to cleave PAR-1 at a different site than thrombin, producing a ligand with a predicted lower affinity for the PAR-1 active site than the classic thrombin-produced ligand.¹⁸ The longer lasting MAPK signaling produced by MMP-1 may also explain the differential induction of gene expression by MMP-1 and thrombin (Table 1), as duration and strength of signaling can effect gene expression.^{56,57} Also, we only examined a subset of pathways activated by PAR-1 cleavage; it is possible that due to the different activating ligands, MMP-1 activates signaling pathways through PAR-1 that thrombin cannot, and vice versa.

The lower affinity activating ligand produced by MMP-1 may also be biologically relevant. Thrombin was shown to strongly inhibit tube formation and branching morphogenesis at high concentrations (Figure 5), while equal amounts of MMP-1 continued to promote angiogenesis. However, like thrombin, MMP-1 signaling through PAR-1 may have a bimodal effect at very high concentrations. For example, Davis and colleagues have demonstrated that a strong induction of MMP-1 expression in endothelial cells causes tube regression in an *in vitro* model.^{58,59} They stimulated cells with phorbol ester, a potent inducer of MMPs, and required 20-fold more MMP-1 neutralizing antibody than we used to completely block MMP-1 activity,⁵⁹ indicating that MMP-1 was at a very high concentration in their system, where it was found to be anti-angiogenic. Also, we have found that the amount of tube formation and branching induced by MMP-1 begins decline at >25 nmol/L (Figure 5), again suggesting that MMP-1 may have bimodal activity, inducing angiogenesis at lower concentrations and preventing it at very high concentrations. However, it is important to note that even at 100 nmol/L, MMP-1 induced significantly more tube formation and branching morphogenesis than PBS control. Such a high concentration in the tumor microenvironment would allow MMP-1 to have collagenolytic activity while maintaining a pro-angiogenic function via PAR-1. This combination would strongly promote tumor progression.

Thrombin is also frequently present in the tumor microenvironment, and immunohistochemical studies have shown the presence of thrombin in melanoma tumor samples.^{52,60} Importantly, we found that the addition of thrombin to media conditioned by a human melanoma cell line, which already produces high levels of MMP-1, induced more angiogenesis than conditioned media alone (Figure 6). These results suggest that the combination of thrombin and MMP-1 may be more pro-angiogenic than either protease alone. Importantly, thrombin could not compensate for the loss of MMP-1 activity in the media (Figure 6), again demonstrating that MMP-1 and thrombin have different effects on endothelial cells, and that one protease cannot completely replace the function of the other.

Thrombin has been shown to promote tumor progression by signaling through PAR-1 on endothelial cells, platelets, myofibroblasts, immune cells and tumor cells, with very diverse downstream effects that are dependent on cell type.^{11,14,52,55} In this study, we have described the induction of signaling pathways and gene expression by MMP-1 activation of PAR-1 in a human microvessel endothelial cell line; however, it is probable that the effects of MMP-1, like thrombin, will vary based on cell type. For example, Boire et al showed that MMP-1 activation of PAR-1, expressed by a human breast cancer cell line, promotes tumor cell invasion⁷; however, we found no change in the invasive potential of HMVECs treated with MMP-1 (data not shown). Thus, it will be important to extend these studies to other cell types present in the tumor microenvironment to understand the full contribution of MMP-1 to tumor progression.

In summary, we have defined a mechanism to explain the recent observations correlating MMP-1 expression and angiogenesis. We have shown that the interstitial collagenase MMP-1 is capable of inducing the expression of a subset of pro-angiogenic genes in human microvessel endothelial cells, and that activation of PAR-1 by thrombin and MMP-1 produce different, but potentially complementary, effects on endothelial cells. As a whole, these findings are suggestive of a new biological role for MMP-1 as a pro-angiogenic signaling molecule.

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