Published in final edited form as:

Exp Eye Res. 2011 November ; 93(5): 618–627. doi:10.1016/j.exer.2011.07.008.

Maspin increases extracellular plasminogen activator activity associated with corneal fibroblasts and myofibroblasts

Debra J. Warejcka^{a,d}, Malathi Narayan^{a,c}, and Sally S. Twining^{a,b,e}

^aDepartment of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

^bDepartment of Ophthalmology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

Abstract

Maspin, an inhibitor of cell migration and a stimulator of adhesion of cells to the ECM, is synthesized and released by corneal keratocytes into the extracellular matrix. When the cornea is wounded, the quiescent stromal keratocytes underlying the wound undergo apoptosis and cells adjacent to this apoptotic area convert to fibroblasts or myofibroblasts. This study explores the effect of extracellular maspin on the plasminogen-plasminogen activator system of corneal stromal cells following wounding. Treatment of corneal fibroblasts and myofibroblasts with r-maspin increased extracellular but not cell-associated tissue-type plasminogen activator (tPA), urinary-type plasminogen activator (uPA) or plasminogen activator inhibitor-1 (PAI-1). Despite the extracellular increase in PAI-1, the net effect of maspin treatment was an increase in plasminogen activation. At physiological levels, maspin did not alter uPA or tPA mRNA levels, in these cells. The increase in pro and active uPA was due to decreased clearance in the presence of maspin for myofibroblasts but not for fibroblasts. The clearance of pro and active tPA was normal in fibroblasts indicating different mechanisms for the increase of these homologous enzymes in the two cell types. Increased generation of plasmin by maspin treated corneal stromal fibroblasts and myofibroblasts led to conversion of plasminogen to active plasmin degradation products and angiostatin-like molecules. This study suggests that extracellular maspin increased pro and active uPA and tPA released by corneal fibroblasts and myofibroblasts on the short time scale of 1–4 hours, but by 24 hrs there was no increase over the levels produced without maspin. This augmentation of plasminogen activator activity increases plasmin activation and angiostatin generation. It further indicates that the effect of maspin on uPA and tPA levels is cell type dependent.

Keywords

Maspin; cornea; turnover; urinary-type Plasminogen Activator; tissue-type Plasminogen Activator; Plasminogen Activator Inhibitor-1; fibroblast; myofibroblast

© 2011 Elsevier Ltd. All rights reserved.

^dwarejck@mcw.edu. ^eCorresponding Author: Sally S. Twining, PhD, Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, stwining@mcw.edu, Phone: 414-955-8431, Fax: 414-955-6050.

^cPresent Address: 625 Light Hall, Vanderbilt University, Nashville, TN 37232, malathi.narayan@Vanderbilt.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Maspin, a non-inhibitory member of the serine proteinase inhibitor (serpin) family, is expressed by most epithelial cells and regulates cell adhesion, migration, and invasion (Ngamkitidechakul et al., 2001; Seftor et al., 1998; Zou et al., 1994). It regulates migration and adhesion of corneal stromal fibroblasts, angiogenesis of vascular endothelial cells and metastasis of carcinoma cells (Ngamkitidechakul et al., 2003; Zhang et al., 2000; Zou et al., 1994). Maspin is synthesized by the three major cells of the cornea; keratocytes and the epithelial and endothelial cells (Narayan and Twining, 2010; Ngamkitidechakul et al., 2001). It is found associated with the cytoplasm and nucleus of the cells and is released from these cells, being found in tears and conditioned medium of keratocytes and epithelial cells (de Souza et al., 2006; Horswill et al., 2008). The stromal layer contains approximately 4× the amount of maspin estimated to be present in the keratocytes (Ngamkitidechakul et al., 2001) suggesting maspin is present in the extracellular matrix (ECM) of the stroma and is available during corneal wound healing.

Corneal wound healing involves multiple processes with the goal of retention or restoration of transparency. Non-injured epithelial cells migrate to recover the wounded area of the corneal surface. These cells release proteins which can enter the stroma through breaks in the basement membrane/Bowman's layer and modulate stromal wound healing. IL-1 released by these cells can regulate fibroblast function (West-Mays et al., 1997) and TGF- β can convert fibroblasts to myofibroblasts and cause fibrosis (Stramer et al., 2003). In addition, maspin is released from the epithelial cells following wounding (Horswill et al., 2008) and may influence the wound healing cells of the stroma.

Stromal keratocytes near the injury site undergo apoptosis and necrosis forming an acellular zone (Mohan et al., 2003). Maspin may be released by dead or dying cells into the extracellular matrix during this process. Keratocytes adjacent to the acellular zone divide and undergo a phenotypic change to wound-associated fibroblasts which are more mobile than the parent keratocytes (Mohan et al., 2003). Maspin synthesis is downregulated by an epigenetic mechanism upon this phenotypic change in the stromal cells (Horswill et al., 2008). The fibroblasts migrate into the acellular zone where they may differentiate into myofibroblasts when they encounter TGF- β from the wounded corneal epithelial cells. Like fibroblasts, myofibroblasts do not synthesize maspin. Fibroblasts and myofibroblasts repopulate the acellular zone and remodel the ECM (Ishizaki et al., 1994; Jester et al., 1995).

Migration of fibroblasts into the acellular zone of the wounded cornea and remodeling of the extracellular matrix are mediated by multiple factors including cell membrane associated and secreted proteases such as matrix metalloproteinases (MMPs), plasminogen activators, urinary (uPA) and tissue type (tPA) and plasmin (Bailey et al., 2009; Bernstein et al., 2004; Fini, 1999; Mohan et al., 2003). Although uPA and tPA share plasminogen activating activity, they have some overlapping and some distinct functions (Binder et al., 2007). uPA is the major plasminogen activator bound to cells via urinary plasminogen activated receptor (uPAR) and is associated with cell invasion of tissues. The major role of tPA is to activate plasminogen for the degradation of fibrin due to its binding affinity for extracellular fibrinogen and fibrin. This is important for degradation of fibrin deposited in the stromal matrix early in the wound healing process (Kao et al., 1998). Failure to remove fibrin from the cornea leads to abnormal delayed wound healing (Drew et al., 2000; Kao et al., 1998).

Most uPA synthesized by corneal fibroblasts is localized to the membrane where it is bound to its receptor uPAR (Bernstein et al., 2007). In contrast, most of the tPA produced by these cells is released into the extracellular milieu. Although myofibroblasts, like fibroblasts, synthesize uPA, this enzyme is released and is not associated with the cell membrane of

myofibroblasts due to degradation of uPAR from the cell surface by a chymotrypsin-like protease (Bernstein et al., 2007). Plasminogen activators convert plasminogen associated with the cell membrane or present in the ECM to plasmin (Binder et al., 2007). This protease activates MMPs from the zymogen form to the active form and directly cleaves ECM proteins and proteoglycans including its major target fibrin (Carmeliet et al., 1997; Ishizaki et al., 1994; Matrisian, 1992; Woessner, Jr., 1991). The plasminogen activator-plasmin system is controlled by three inhibitory serpins, plasminogen activator inhibitors (PAI) 1 and 2 and α_2 -antiplasmin, inhibitors present in the cornea (Ayala et al., 2005; Berk et al., 2001; Ottino et al., 2002).

During the wound healing process, fibroblasts and myofibroblasts would encounter maspin present in the extracellular matrix of the cornea ($\sim 2.5 \mu\text{M}$) released from resident keratocytes prior to wounding and upon wounding from migrating epithelial cells (Horswill et al., 2008) through basement membrane breaks and keratocytes undergoing cell death (Ngamkitidechakul et al., 2001). This is in contrast to a recent study on MCF10A mammary epithelial cells that suggests that maspin is not released from these cells in culture (Teoh et al., 2010). Although maspin does not possess a classical signal sequence, mechanisms other than the classical ER-golgi route probably account for maspin release by corneal cells.

In the cornea, interaction of maspin with corneal stromal fibroblasts and myofibroblasts may alter cell migration and ECM remodeling through regulation of the plasminogen activator/plasminogen system. The effects of maspin on this system have previously been studied in DU145 prostate carcinoma cells and MDA-MB-231 mammary carcinoma cells (Amir et al., 2005; McGowen et al., 2000). Cell associated uPA was decreased by exogenous maspin or stable transfection of the maspin gene into the carcinoma cells. Secreted uPA was decreased by transfection of the maspin gene but not by exogenously added maspin (Amir et al., 2005; Biliran, Jr. and Sheng, 2001; McGowen et al., 2000). Maspin has no direct effect on the activity of uPA or tPA but does form complexes with these proteases at a site other than the reactive site loop (Al-Ayyoubi et al., 2007; Bass et al., 2002).

The current study explores the interaction of extracellular maspin with non-transformed primary corneal stromal fibroblasts and myofibroblasts. We hypothesize that these cell types, though no longer producing maspin, would still be regulated by the maspin in the extracellular milieu.

2. Experimental

2.1. Human corneas

Human corneas were obtained from the Wisconsin Lions Eye Bank (Madison, WI) or The National Disease Research Interchange within 24–48 hours of death. Experiments with corneas followed the tenets of the Declaration of Helsinki (1989) of the World Medical Association. All experiments were carried out using cells from at least three different donors.

2.2. Corneal Stromal Cell Culture

After scraping the endothelial and epithelial layers from the donor corneas, the stromal cells were released by collagenase digestion as previously described by Taylor et al (Taylor et al., 1989). The cultured stromal cells were maintained in high glucose DMEM supplemented with 1% L-glutamine (Invitrogen) with 5% defined FBS (Hyclone), 0.1% MITO⁺ serum extender (Becton Dickinson) and 10 $\mu\text{g/ml}$ ciprofloxacin (Sigma) at 37° C in 5% CO₂. Fibroblastic and myofibroblastic phenotypes were generated from the P4 and P5 stromal cell populations as described by Jester (Petroll et al., 1996). Briefly, the stromal cells were seeded onto collagen-coated (Inamed) wells in Defined Medium (DMEM plus 1% RPMI

vitamin mix (Invitrogen), 100 μ M non-essential amino acids, 1 mM pyruvate, 100 μ g/ml ascorbic acid, and 10 μ g/ml ciprofloxacin). For fibroblasts, 10 ng/ml fibroblast growth factor-2 (FGF-2) (Invitrogen) was added to the medium. For myofibroblasts, 1 ng/ml transforming growth factor- β 1 (TGF β 1) (R&D Systems) was added to the medium. Cells were maintained for seven days in growth factor-containing medium before starting any experiment.

2.3. Maspin

Recombinant maspin was produced in yeast and purified as previously described (Ngamkitidechakul and Twining, 2002). The preparations of maspin were tested for the presence of lipopolysaccharide (LPS) using the Limulus amoebocyte lysate assay (Cambrex Bio Science) and confirmed to be negative. The recombinant maspin was also negative for protease activity including the ability to activate plasminogen using gelatin and casein zymography and the FITC casein assay in the presence and absence of plasminogen (Warejcka et al., 2005; Twining, 1984). Cultures of fibroblasts and myofibroblasts were treated for various time periods with 1 μ M maspin, well within physiological levels (Ngamkitidechakul et al., 2001).

2.4. Conditioned Medium

At the time points noted in the results, medium was removed from maspin treated and control cells, spun in a microfuge to remove cell fragments and frozen at -20°C until use. Protein is not detectable in the conditioned medium from cells so to normalize loading for SDS-PAGE, cells corresponding to the conditioned medium were solubilized with 0.5 M NaOH and the amount of protein was determined using Pierce Coomassie Plus assay. Conditioned medium was normalized to the amount of cellular protein in the well which is in turn a reflection of cell number.

2.5. Cell Lysates

Cell monolayers were washed twice with PBS and incubated at 4°C for 20 minutes in detachment buffer (250 mM sucrose, 10 mM Tris pH 7.6, 1 mM EDTA, 1X protease inhibitor cocktail (Roche Complete) plus 1 mM PMSF) then scraped from the plastic with a rubber policeman. After pelleting the cells, lysis buffer (150 mM NaCl, 10 mM Tris pH 7.6, 1 mM EDTA, 1% Triton X-100, 0.05% SDS, 1X protease inhibitor cocktail (Roche Complete) plus 1 mM PMSF) was added to the pellet and the cells were vortexed intermittently and kept on ice for 30 minutes and then frozen at -20°C . Thawed lysates were centrifuged and the supernatants were assayed for protein content using Coomassie Plus. (Pierce).

2.6. Western Blots

Conditioned media from stromal cells were normalized for the corresponding cellular protein levels and separated on 10% SDS polyacrylamide gels under non-reducing conditions. The proteins were transferred to nitrocellulose membranes (BioRad). The blots were blocked with 10% (w/v) nonfat dry milk (Nestle USA, Inc., Solon, OH), washed and probed as previously reported (Twining et al., 1994). The primary mouse monoclonal antibodies were specific for PAI-1 (Calbiochem), maspin (BD Biosciences), PAI-2, α 2-antiplasmin, plasminogen and uPAR (American Diagnostica). The secondary antibody was horseradish peroxidase conjugated goat antimouse IgG (BioRad). The specific bands were visualized on film (Hyperfilm, Amersham) using a luminol based chemiluminescence system (ECL, Amersham).

2.7. Plasminogen Activation Assay

Plasminogen activator activity was measured indirectly by quantifying plasmin activity using a FITC-labeled casein assay (Twining, 1984). Plasmin activity in conditioned medium from fibroblasts and myofibroblasts was measured by adding an equal volume of FITC-casein +/- plasminogen (0.17 μ M) to conditioned media and incubating for 24 hours. Undigested casein was precipitated with 5% TCA and removed by centrifugation. The supernatant fractions were neutralized with 0.5 M Tris-HCl, pH 8.6 and the fluorescence of samples was read using an excitation of 490 nm and emission of 550 nm. All samples were done in triplicate. Activity measured in samples not containing plasminogen with and without maspin (background) was subtracted from the activity measured in plasminogen containing samples. To measure cell associated plasminogen activator activity, medium was aspirated from cells and replaced with FITC-labeled casein in DMEM with or without plasminogen. Maspin (1 μ M) was added back to the cultures that originally were incubated with this protein. After 24 hours, conditioned medium was removed from cells and treated as above.

2.8. Zymograms

Conditioned medium or cell lysates were normalized for cellular protein, separated on non-reducing 10% SDS-PAGE containing 0.2% casein for plasmin detection or 0.2% casein plus plasminogen (Haematologic Technologies, Inc) for plasminogen activator detection. The bands were developed and visualized as previously reported (Warejcka et al., 2005). The bands on the casein-plasminogen gels for uPA and tPA represent both active (two chain) and pro (one chain) forms because the two chain forms are activated during gel processing (Corti et al., 1986; Grau and Moroz, 1989; Ismail et al., 2006). The bands on some gels were digitized using ImageJ (National Institutes of Health) and plotted using Prism (GraphPad).

2.9. Cycloheximide treatment

After phenotyping the stromal cells for one week in growth factors (FGF-2 or TGF β), cells were fed with medium containing 10 μ g/ml cycloheximide (Sigma) and incubated for 16 hours. Then medium containing 10 μ g/ml cycloheximide +/- maspin was added to cells. Conditioned media and cell lysates were collected from wells after 1, 3, 4 or 6 hours of incubation.

2.10. Angiostatin Generation

Stromal fibroblasts or myofibroblasts were treated with or without maspin for 24 hours. Conditioned media were removed from cells, spun down and aliquots were incubated with or without 33 nM plasminogen (final concentration) for 72 hours. The resulting supernatant samples were separated on 10% SDS-PAGE, transblotted to nitrocellulose and blocked with nonfat dry milk. Blots were probed with a mixture of two antibodies specific for kringle 1–3 of plasminogen (American Diagnostica and Enzyme Research Laboratories, Inc).

2.11. Real Time-PCR

RNA from fibroblasts and myofibroblasts was collected and purified using the RNEasy kit (Qiagen). One μ g of RNA was used to prepare cDNA using Superscript III (Invitrogen). The SYBR Green Master Mix (SABiosciences) was used to perform polymerase chain reaction (PCR) using the Q-PCR Stratagene MX3005 instrument. The primers from IDT were the following:

Forward: 5'-AGCTGTGAGATCACTGGCTTTGGA-3' and

Reverse: 5'-AGTCTCCCTGGCAGGAATCTGTTT-3' for uPA,

Forward: 5'-AAGCAACCGGGTGGATATTGCTG-3' and

Reverse: 5'-AGCACGTGGCCCTGGTATCTATTT-3' for tPA and

Forward: 5'-TCGACAGTCAGCCGCATCTTCTTT-3' and

Reverse: 5'-ACCAAATCCGTTGACTCCGACCTT-3' for GAPDH.

Data was calculated using the MXPro software (Stratagene) to determine amounts of mRNA relative to the fibroblast control after correcting for GAPDH content.

2.12. Statistical Analysis

Prism 5.02 (Graph Pad) was used to analyze numerical data from the FITC-casein degradation experiments and the mRNA quantification experiments. The effect of maspin at one time point on either the fibroblasts or the myofibroblasts was analyzed using the two tailed paired t-test. The effect of maspin over time on the two cell types was analyzed using a two-tailed ANOVA followed by individual comparisons using the Bonferroni test. mRNA levels were analyzed using the Student's t-test.

3. Results

3.1. Maspin increases extracellular pro and active forms of plasminogen activators

To determine the effects of maspin on plasminogen activators secreted by stromal fibroblasts and myofibroblasts, casein-plasminogen zymography was performed on conditioned medium from cells incubated in the presence and absence of maspin. Human donor corneal stromal fibroblasts and myofibroblasts were treated with physiological levels of maspin present in the extracellular matrix of the corneal stroma (Ngamkitidechakul et al., 2001). Casein-plasminogen zymography was used to determine the total pro (single chain) and active (two chain, disulfide linked) levels of uPA and tPA. These two forms of uPA and tPA are both active on the zymograms because under the non-reducing conditions used, both forms co-migrate and the single chain forms are activated during processing of the zymogram gels (Corti et al., 1986; Grau and Moroz, 1989; Ismail et al., 2006). With and without physiological levels of maspin (1 μ M), pro and active uPA and tPA accumulated in the conditioned medium from stromal cells over the 24 hour time period tested (Figure 1). No active plasmin or any other enzyme that cleaves casein was detected in the fibroblast or myofibroblast conditioned media with or without maspin as determined by casein zymography (data not shown). At early time points, the conditioned medium from cells incubated with maspin had higher levels of plasminogen activators than that of the control cells (Figure 1), whereas the cell associated activators were not affected by the presence of maspin (Figure 2).

The major plasminogen activator present in fibroblast conditioned medium was tPA, which increased upon maspin treatment at 1 and 4 hrs (Figure 1). Pro and active forms of uPA were low in the fibroblasts at all time points but a greater amount was observed in the presence of maspin. The amount of pro and active uPA in the conditioned media was donor dependent. This uPA probably represents an amount that exceeds the binding capacity of uPAR on the surface of these cells.

The major plasminogen activator observed in myofibroblast conditioned medium was uPA (Figure 1). The pro plus active forms of this activator were increased upon maspin treatment at 1, 4 and 8 hrs. A low amount of pro and active forms of tPA was observed in the myofibroblast conditioned medium produced in the presence of maspin at one hour but was not observed until later in the control cultures. This tPA may be produced by a very small number of contaminating fibroblasts (<1%) because the amount of tPA in cultures from

different donors can vary from none to a small amount. Similar results for the fibroblasts and myofibroblasts were observed on western blots for uPA and tPA (data not shown). These results suggest that maspin affects the regulation of extracellular plasminogen activators by stromal cells.

3.2. Maspin increases levels of PAI-1

Because plasminogen activation depends on the relative amount of plasminogen activator inhibitors to the amount of plasminogen activators present, the effect of maspin treatment of the corneal stromal fibroblasts and myofibroblasts on the levels of PAI-1 and PAI-2 present in the conditioned media of these cells was determined. Maspin increases PAI-1 levels in corneal stromal fibroblast and myofibroblast conditioned medium at 4 and 8 hrs (Figure 3). The observed single PAI-1 band represents intact, active protein which migrates distinctly slower than the inactive cleaved or latent (altered conformation) forms. This protein accumulated over the 24 hr period in the presence and absence of maspin. The levels of PAI-1 in the conditioned medium of corneal stromal fibroblasts and myofibroblasts varied greatly between donors but in all cases ($n = 8$) maspin increased PAI-1 levels at early time points (4–8 hrs). Because PAI-1 synthesis is very sensitive to the presence of LPS, the maspin preparations were tested and found to be negative for LPS (less than 0.06 EU/ml). The preparations were free of contaminating proteases. Intracellular PAI-1 levels were very low in all cell types as expected (data not shown) because this inhibitor contains a classical signal sequence and is mostly secreted (Binder et al., 2002). Like maspin, the second plasminogen activator inhibitor, PAI-2, does not contain a classical secretory sequence (Ritchie and Booth, 1998).

Although some cells release a second plasminogen activator inhibitor, PAI-2 into the extracellular milieu, corneal stromal fibroblasts and myofibroblasts in the presence and absence of maspin do not release PAI-2. This inhibitor is exclusively cell associated in the corneal fibroblasts or myofibroblasts and is not affected by maspin (data not shown). These results suggest that maspin increases PAI-1 levels in the medium of corneal stromal cells but does not affect cellular levels of either PAI-1 or PAI-2.

3.3. Maspin increases net extracellular plasminogen activation

Since conditioned medium from maspin treated fibroblasts and myofibroblasts contained increased levels of the pro and active forms of plasminogen activators, uPA and tPA (Figure 1), and the inhibitor, PAI-1 (Figure 3), we determined the effect of maspin treatment of the cells on net plasminogen activator activity using a solution based coupled plasminogen-FITC-casein assay. This assay measures only the activated forms of plasminogen activators. Plasminogen was added in these experiments because although plasminogen is synthesized by human corneal keratocytes and is present in the normal human cornea, it is not secreted by corneal fibroblasts and myofibroblasts in culture (Twining et al., 1999). Conditioned medium from maspin treated stromal fibroblasts and myofibroblasts increased plasminogen activation and casein cleavage relative to the control medium produced in the absence of maspin (Figure 4 A, B). The greatest difference in the net activity was at 24 hrs with significant differences ($p < 0.01$) observed at 8 and 24 hrs for both cell types in the presence and absence of maspin. In the absence of conditioned medium, plasminogen was not activated (data not shown). To further explore the effect of maspin on plasminogen activators in the conditioned medium from corneal stromal fibroblasts and myofibroblasts, additional donors were tested. The increase in the active forms of plasminogen activator in the presence of maspin was observed for all 6 of the additional fibroblast donors and 5 of the 6 additional myofibroblast donors (Figure 4C, D). Analysis of these results using a two-tailed paired t-test revealed the maspin significantly ($p < 0.005$) increased plasminogen activator activity over the control for both the fibroblasts and myofibroblasts. The net

activity probably was influenced by the maspin stimulated increase in PAI-1 levels, but the inhibitors present were not great enough to balance the increase in plasminogen activators. The corneal stromal fibroblasts and myofibroblasts synthesize mRNA for α_2 -antiplasmin (Ayala et al., 2007) an inhibitor that would influence the measured net plasmin activity. Supernatants were probed by western blot for the presence of this protease inhibitor. No α_2 -antiplasmin was detected (data not shown). These experiments suggest the net effect of maspin on the extracellular plasminogen activator system in corneal stromal fibroblasts and myofibroblasts is to increase plasminogen activator activity, and the increase in extracellular PAI-1 in the presence of maspin does not overcome the increase in the plasminogen activators.

To confirm that conditioned medium from maspin treated fibroblasts and myofibroblasts stimulated an increase in the active full length and cleavage forms of the plasmin, 24 hr media conditioned by corneal stromal fibroblasts or myofibroblasts in the presence and absence of maspin was incubated with plasminogen for an additional 24 hrs and the proteins in the media were separated by casein zymography. This type of zymography detects active plasmin and its active cleaved forms but not plasminogen activators or plasminogen. In the absence of plasminogen, conditioned media did not degrade casein (Figure 5, Lanes 1, 3, 5 and 7). Plasminogen incubated with non-conditioned medium was not activated to plasmin (data not shown). Conditioned media from cells treated with maspin and then incubated in the presence of plasminogen contained more active full length plasmin and plasmin degradation products than conditioned media from cells incubated in the absence of maspin (Figure 5, Lanes 2 vs 4 for fibroblasts and 6 vs 8 for myofibroblasts). These results show that the presence of maspin stimulates the formation of both full length and active cleaved forms of plasmin.

3.4. Maspin does not alter membrane associated plasminogen activator activity

Proteolysis of the ECM is one of the major mechanisms involved in migration of cells (Werb, 1997). Plasminogen activators bound at the leading front of migrating cells activate plasminogen to plasmin leading to extracellular matrix degradation. To determine whether maspin altered plasminogen activation at the cell membrane of intact corneal stromal fibroblasts and myofibroblasts, the cells were cultured with and without maspin for increasing times. The conditioned medium was removed at various times up to 24 hrs and the cells were further incubated in the presence of plasminogen and FITC-labeled casein in DMEM with and without maspin for 24 hrs. This medium without FGF-2 and TGF- β and maintained the cells under isotonic conditions but did not support proliferation. As expected, based on the presence of uPA bound to its receptor uPAR (Bernstein et al., 2007), corneal fibroblasts efficiently generated plasmin that degraded FITC-casein (Figure 6A). The presence of maspin did not significantly alter cleavage of FITC-casein by the intact corneal fibroblasts. Consistent with these results, western blots performed on lysates of fibroblasts cultured in the presence and absence of maspin showed no significant difference in total uPAR, the major plasminogen activator receptor on the fibroblast cell membrane. (Figure 6B). Because of the absence of intact uPAR on the surface of myofibroblasts (Bernstein et al., 2007), essentially no cleavage of FITC-casein was observed for these cells (Figure 6A). The presence of maspin did not increase the cleavage of FITC-casein by the myofibroblasts. These experiments indicate that maspin does not significantly alter the net level of membrane-bound plasminogen activators present on the corneal fibroblasts or myofibroblasts.

3.5. Maspin does not affect uPA or tPA mRNA levels

One mechanism by which plasminogen activators could be increased in the conditioned media from maspin treated corneal stroma derived fibroblasts and myofibroblasts is through

increased gene expression and subsequent protein synthesis. To test this possibility the relative levels of mRNA in the cells for uPA and tPA were determined. Initial semi-quantitative RT-PCR experiments showed no differences in mRNA levels at time points from 1–24 hrs (Data not shown). For more quantitative experiments using SYBR Green and real time-PCR, one hour of incubation with maspin was chosen for three additional donors because the greatest changes in secreted uPA and tPA in response to maspin were observed at 1 and 4 hours (Figure 1). As would be predicted from the pro and active levels, the mRNA level for tPA normalized to GAPDH was much greater for the corneal stromal fibroblasts than for the myofibroblasts (Figure 7A). The level of mRNA for uPA normalized to GAPDH in myofibroblasts was about double that in fibroblasts (Figure 7B). These relationships held for mRNA levels of the plasminogen activators from these cells cultured in the presence of maspin. No significant differences, as determined by the two-tailed paired t-test, were observed in the mRNA levels for uPA or tPA in either fibroblasts or myofibroblasts cultured with or without maspin treatment (Figure 7A, B) despite an increase in the translated products of these genes in the three sets of cultures from which the mRNA was extracted (data not shown). This suggests the difference in the levels of pro and active plasminogen activators in the conditioned media is not due to increased transcription but to alterations in protein synthesis, secretion and/or stability.

3.6. Maspin induces increased stability of extracellular pro and active uPA from myofibroblasts, but not pro and active tPA nor uPA from fibroblasts

To determine whether maspin influences the stability and uptake of uPA and tPA, cells were treated with cycloheximide to inhibit protein synthesis including proteins secreted into the medium. Thus, loss of uPA and tPA from the medium is due to uptake of the activators by the cells or degradation in the medium. Zymography was performed on the conditioned medium from cells treated with or without cycloheximide and with or without maspin. In the experiment depicted in Figure 8, control fibroblasts made and secreted pro and active tPA (Figure 8A upper bands, left lanes, 8B lower curve) and upon maspin treatment, the amount of pro and active tPA in conditioned medium collected after 1, 3, 4 and 6 hours was increased relative to the conditioned medium from untreated cells (Figure 8A upper bands, right lanes vs left lanes, Figure 8B, upper vs lower curve). Under both conditions, pro and active tPA at 6 hrs was greater than at 1 hr (Figure 8A upper bands and Figure 8B). When fibroblasts were treated with cycloheximide, the relative amount of pro and active tPA decreased (Figure 8D, upper bands, left lanes, Figure 8E, lower curve) as expected because translation was inhibited. This decrease in the amount of pro and active tPA over 4 hours suggests the tPA was taken up by the cells or degraded in the medium. Addition of maspin to cycloheximide cultures resulted in an increase of tPA, but the rate of disappearance of tPA from the medium over four hours closely mirrored that of the control cycloheximide culture (Figure 8D, upper bands, right lanes vs left lanes, Figure 8E, upper curve vs lower curve) signifying that the uptake or degradation of pro and active tPA was not changed by the presence of maspin.

Fibroblasts also synthesize and secrete pro and active uPA and this amount is increased upon treatment of the cells with maspin (Figure 8A, lower bands, right vs left bands, Figure 8C, upper vs lower curve). When the translation of proteins was inhibited with cycloheximide, there was less pro and active uPA as expected and, as with the tPA, the pro and active uPA disappeared from the medium at 1–4 hours as it was taken up by cells or degraded in the medium (Figure 8D, lower bands, left lanes, Figure 8F, lower curve). Maspin did not change this uptake mechanism (Figure 8D, lower bands, right lanes vs left lanes, Figure 8F upper vs lower curve).

Myofibroblasts in control medium synthesize pro and active uPA and when treated with maspin, the amount of pro and active uPA was increased at 1, 3 and 4 hrs but not at 6 hrs

(Figure 8G, right vs left lanes, Figure 8H, upper vs lower curve). When myofibroblasts were treated with cycloheximide to inhibit translation of proteins, the amount of pro and active uPA in the medium decreased (Figure 8I, left lanes, Figure J, lower curve). Over 4 hours the amount of pro and active uPA in the medium decreased as the uPA was cleared by cell uptake or inactivated in the medium. When maspin was added to the cycloheximide cultures, the amount of pro and active uPA remained relatively stable over 4 hours with an increase at 6 hrs of culture (Figure 8I, right lanes, Figure 8J, upper curve). This suggests that maspin inhibits the uptake and/or inactivation of pro and active uPA by the myofibroblasts resulting in higher levels of pro and active uPA in the conditioned medium of maspin treated myofibroblast cultures.

3.7. Maspin increases angiostatin generation

The increase in pro and active uPA and tPA in the presence of maspin would suggest increased proteolysis of proteins in the extracellular matrix. One of these molecules is plasminogen which can be activated and converted to angiostatin, an antiangiogenic molecule which upon depletion results in neovascularization of the cornea (Gabison et al., 2004). In the present experiments, the increased levels of active plasmin degradation products (Figure 5) suggest angiostatin, a cleavage product of plasminogen with antiangiogenic activity, may also be increased in the presence of maspin. Conditioned medium from fibroblasts and myofibroblasts treated with and without maspin were incubated with plasminogen, separated on SDS-PAGE and western blots were performed to detect angiostatin. Although fibroblast angiostatin production is observed at 24 hrs (Warejcka et al., 2005), the current experiments were carried out for 72 hrs to maximize the products observed. Samples from cells treated with maspin contained more of the larger form of K1-4 angiostatin than the control samples (Fig. 9). Without maspin, myofibroblasts produce very low levels of angiostatins. These data suggest that maspin can induce an increase in the concentration of an anti-angiogenic molecule, angiostatin, in the cornea during wound healing.

4.0. Discussion

Exogenous maspin, at physiological levels for the cornea, increases the levels of the pro and active forms of plasminogen activators, uPA and tPA and their inhibitor, PAI-1, in the extracellular milieu of cultured corneal fibroblasts and myofibroblasts but does not affect cell associated levels of these components of the plasminogen/plasminogen activator system. Despite the increase in PAI-1, the net effect of maspin was to increase plasminogen activation. These results differ from those previously reported for the effect of exogenous maspin on DU145 prostate carcinoma cells (McGowen et al., 2000). Addition of maspin to cultures of these carcinoma cells decreased cell associated uPA and did not affect the extracellular levels of this plasminogen activator. This difference is not due to differences in maspin between laboratories. Our recombinant maspin made in yeast possesses the same biological activity observed by others; it increases adhesion and inhibits invasion of MDA-MB-231 breast carcinoma cells (Ngamkitidechakul et al., 2003). In addition, our recombinant maspin does not increase pro and active uPA present in the MDA-MB 231 breast carcinoma cell conditioned medium (data not shown). This is consistent with that reported for DU145 cells exogenously treated with maspin (McGowen et al., 2000). The distinct effects of maspin on uPA levels in conditioned medium of the breast and prostate carcinoma cells relative to that on the corneal cells may be due to basic differences between the transformed epithelioid and normal fibroblastic cell types. The physiological roles of the corneal fibroblasts relative to the carcinoma cells may determine the repertoire of genes expressed and thus modify the response of the different cell types to maspin. Upon injury, remodeling of the cornea is a normal physiological process that involves the activation of

plasminogen to plasmin. An increase in uPA and tPA by maspin may be important in the remodeling of the injured tissue. These experiments suggest maspin can interact with cells and affect specific functions in a cell specific manner.

The mechanisms of the increase in pro and active uPA and tPA in fibroblast and myofibroblast conditioned media differ. In the presence of maspin, pro and active tPA and uPA from cycloheximide-treated fibroblasts was taken up by cells or inactivated normally while the clearance of pro and active uPA in cycloheximide-treated myofibroblasts was inhibited. tPA and uPA bind to different receptors on the cell surface. Most notable is the binding of uPA to uPAR which can mediate turnover of uPA through interaction with other receptors such as low density lipoprotein receptor-related protein (LRP) or the insulin-like growth factor II receptor 2 (IGFII-R2) (Conese et al., 1995; Nykjaer et al., 1998). uPAR may be involved in the observed difference in the rate of clearance of pro and active uPA by myofibroblasts in the presence of maspin because maspin affects turnover of uPA in myofibroblasts but not fibroblasts. Fibroblasts but not myofibroblasts have intact uPAR on their cell surfaces (Bernstein et al., 2007). A separate mechanism must be involved for tPA. Experiments using uPAR^{-/-} cells revealed uPA interacts with at least one other receptor on the cell surface and can initiate signaling pathways (Zhang et al., 2004). A possible receptor is the nicotinic receptor-1 α which upon binding uPA on renal fibroblasts induces a signaling cascade that leads to conversion of the fibroblasts to myofibroblasts and stimulation of fibrosis (Zhang et al., 2009). There is the possibility that a similar mechanism occurs on corneal fibroblasts. Due to a shortened hinge region, maspin does not form the classic stable serpin inhibitory complexes involving the reactive site loop of maspin and the active site of target proteases. However, maspin can form complexes with uPA and tPA which do not involve the RSL of maspin nor the active sites of the activators (Al-Ayyoubi et al., 2007). It is possible that formation of such a complex prevents binding of uPA to a receptor on the myofibroblast surface and limits uptake of uPA. Alternatively, maspin may alter a pathway that is involved in the turnover of uPA in myofibroblasts but not fibroblasts.

Maspin is known to inhibit angiogenesis in the rat corneal pocket model (Zhang et al., 2000). It also inhibits migration and tube formation of vascular endothelial cells. The current experiments suggest an additional indirect mechanism of inhibition of neovascularization. The importance of this mechanism of generation of angiostatin awaits further experimentation; however, angiostatin represents a crucial anti-angiogenic molecule in the balance between pro and anti-angiogenic molecules. Wounding of corneas treated with antibodies to angiostatin results in angiogenesis in contrast to wounding without angiostatin antibodies (Gabison et al., 2004).

In conclusion, the present work suggests maspin can influence the levels of the components of the plasminogen activator system in a cell dependent manner based on the role of the cells within a given tissue. In the present study, exogenously added maspin increased the levels of extracellular active and pro forms of uPA and tPA but not corneal stromal fibroblast or myofibroblast associated uPA and tPA. The mechanism of the maspin induced increase in pro and active extracellular uPA in myofibroblasts may be due to decreased clearance while that for the increase in extracellular pro and active tPA is probably by another mechanism such as coordinated increase in translation and secretion. These results implicate maspin as a regulator of remodeling of the corneal matrix, as well as, controlling cell adhesion during corneal wound healing.

Research Highlights

- Maspin increases plasminogen activators in fibroblast and myofibroblast media.
- Maspin increases PAI-1 levels but the net effect is plasminogen activation.

- Maspin does not alter plasminogen activator mRNA levels.
- Maspin does not alter cell associated plasminogen activator levels.
- Maspin induces increased stability of extracellular myofibroblast uPA, but not fibroblast uPA or tPA.

Abbreviations

DMEM	Dulbecco's Modified Eagle Medium
FGF-2	fibroblast growth factor-2
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LPS	Lipopolysaccharide
PAI-1	Plasminogen Activator Inhibitor
Q-PCR	Quantitative-Polymerase Chain Reaction
rMaspin	recombinant maspin
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TGF	transforming growth factor
tPA	tissue-type Plasminogen Activator
uPA	urokinase-type-Plasminogen Activator
uPAR	urokinase-type-Plasminogen Activator Receptor

Acknowledgments

This study was funded by grants from the Eye Institute of the National Institutes of Health (RO1-EY12731, RO1-14168 and P30-EY01931) and an unrestricted grant from Research for the Prevention of Blindness to the Department of Ophthalmology.

Reference List

1. Al-Ayyoubi M, Schwartz BS, Gettins PG. Maspin binds to urokinase-type and tissue-type plasminogen activator through exosite-exosite interactions. *J.Biol.Chem.* 2007; 282:19502–19509. [PubMed: 17510061]
2. Amir S, Margaryan NV, Odero-Marrah V, Khalkhali-Ellis Z, Hendrix MJ. Maspin regulates hypoxia-mediated stimulation of uPA/uPAR complex in invasive breast cancer cells. *Cancer Biol.Ther.* 2005; 4:400–406. [PubMed: 15846059]
3. Ayala A, Warejcka DJ, Olague-Marchan M, Twining SS. Corneal activation of prothrombin to form thrombin, independent of vascular injury. *Invest Ophthalmol.Vis.Sci.* 2007; 48:134–143. [PubMed: 17197526]
4. Ayala A, Warejcka DJ, Vaughan KA, Twining SS, Yue BY. The fibrinolysis inhibitor alpha2-antiplasmin in the human cornea. *Curr.Eye Res.* 2005; 30:1097–1103. [PubMed: 16354623]
5. Bailey CM, Margaryan NV, Abbott DE, Schutte BC, Yang B, Khalkhali-Ellis Z, Hendrix MJ. Temporal and spatial expression patterns for the tumor suppressor Maspin and its binding partner interferon regulatory factor 6 during breast development. *Dev.Growth Differ.* 2009; 51:473–481. [PubMed: 19527266]
6. Bass R, Fernandez AM, Ellis V. Maspin inhibits cell migration in the absence of protease inhibitory activity. *J.Biol.Chem.* 2002; 277:46845–46848. [PubMed: 12384513]

7. Berk RS, Katar M, Dong Z, Day DE. Plasminogen Activators and Inhibitors in the Corneas of Mice Infected with *Pseudomonas aeruginosa*. *Invest Ophthalmol.Vis.Sci.* 2001; 42:1561–1567. [PubMed: 11381061]
8. Bernstein AM, Greenberg RS, Taliana L, Masur SK. Urokinase anchors uPAR to the actin cytoskeleton. *Invest Ophthalmol.Vis.Sci.* 2004; 45:2967–2977. [PubMed: 15326109]
9. Bernstein AM, Twining SS, Warejcka DJ, Tall E, Masur SK. Urokinase receptor cleavage: a crucial step in fibroblast-to-myofibroblast differentiation. *Mol.Biol.Cell.* 2007; 18:2716–2727. [PubMed: 17507651]
10. Biliran H Jr. Sheng S. Pleiotrophic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. *Cancer Res.* 2001; 61:8676–8682. [PubMed: 11751384]
11. Binder BR, Christ G, Gruber F, Grubic N, Hufnagl P, Krebs M, Mihaly J, Prager GW. Plasminogen activator inhibitor 1: physiological and pathophysiological roles. *News Physiol Sci.* 2002; 17:56–61. [PubMed: 11909993]
12. Binder BR, Mihaly J, Prager GW. uPAR-uPA-PAI-1 interactions and signaling: a vascular biologist's view. *Thromb.Haemost.* 2007; 97:336–342. [PubMed: 17334498]
13. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat.Genet.* 1997; 17:439–444. [PubMed: 9398846]
14. Conese M, Nykjaer A, Petersen CM, Cremona O, Pardi R, Andreasen PA, Gliemann J, Christensen EI, Blasi F. alpha-2 Macroglobulin receptor/Ldl receptor-related protein(Lrp)-dependent internalization of the urokinase receptor. *J.Cell Biol.* 1995; 131:1609–1622. [PubMed: 8522616]
15. Corti A, Nolli ML, Soffientini A, Cassani G. Purification and characterization of single-chain urokinase-type plasminogen activator (pro-urokinase) from human A431 cells. *Thromb.Haemost.* 1986; 56:219–224. [PubMed: 3101222]
16. de Souza GA, Godoy LM, Mann M. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. *Genome Biol.* 2006; 7:R72. [PubMed: 16901338]
17. Drew AF, Schiman HL, Kombrinck KW, Bugge TH, Degen JL, Kaufman AH. Persistent corneal haze after excimer laser photokeratectomy in plasminogen-deficient mice. *Invest Ophthalmol.Vis.Sci.* 2000; 41:67–72. [PubMed: 10634603]
18. Fini ME. Keratocyte and fibroblast phenotypes in the repairing cornea. *Prog.Retin.Eye Res.* 1999; 18:529–551. [PubMed: 10217482]
19. Gabison E, Chang JH, Hernandez-Quintela E, Javier J, Lu PC, Ye H, Kure T, Kato T, Azar DT. Anti-angiogenic role of angiostatin during corneal wound healing. *Exp.Eye Res.* 2004; 78:579–589. [PubMed: 15106938]
20. Grau E, Moroz LA. Fibrinolytic activity of normal human blood monocytes. *Thromb.Res.* 1989; 53:145–162. [PubMed: 2922705]
21. Horswill MA, Narayan M, Warejcka DJ, Cirillo LA, Twining SS. Epigenetic silencing of maspin expression occurs early in the conversion of keratocytes to fibroblasts. *Exp.Eye Res.* 2008; 86:586–600. [PubMed: 18291368]
22. Ishizaki M, Wakamatsu K, Matsunami T, Yamanaka N, Saiga T, Shimizu Y, Zhu G, Kao WW. Dynamics of the expression of cytoskeleton components and adherens molecules by fibroblastic cells in alkali-burned and lacerated corneas. *Exp.Eye Res.* 1994; 59:537–549. [PubMed: 9492755]
23. Ismail B, Choi LH, Were LM, Nielsen SS. Activity and nature of plasminogen activators associated with the casein micelle. *J.Dairy Sci.* 2006; 89:3285–3295. [PubMed: 16899661]
24. Jester JV, Petroll WM, Barry PA, Cavanagh HD. Expression of alpha-smooth muscle (alpha-SM) actin during corneal stromal wound healing. *Invest Ophthalmol.Vis.Sci.* 1995; 36:809–819. [PubMed: 7706029]
25. Kao WW, Kao CW, Kaufman AH, Kombrinck KW, Converse RL, Good WV, Bugge TH, Degen JL. Healing of corneal epithelial defects in plasminogen- and fibrinogen-deficient mice. *Invest Ophthalmol.Vis.Sci.* 1998; 39:502–508. [PubMed: 9501859]
26. Matrisian LM. The matrix-degrading metalloproteinases. *Bioessays.* 1992; 14:455–463. [PubMed: 1445287]

27. McGowen R, Biliran H Jr, Sager R, Sheng S. The surface of prostate carcinoma DU145 cells mediates the inhibition of urokinase-type plasminogen activator by maspin. *Cancer Res.* 2000; 60:4771–4778. [PubMed: 10987285]
28. Mohan RR, Hutcheon AE, Choi R, Hong J, Lee J, Mohan RR, Ambrosio R Jr, Zieske JD, Wilson SE. Apoptosis, necrosis, proliferation, and myofibroblast generation in the stroma following LASIK and PRK. *Exp.Eye Res.* 2003; 76:71–87. [PubMed: 12589777]
29. Narayan M, Twining S. Focus on molecules: maspin. *Exp.Eye Res.* 2010; 90:2–3. [PubMed: 19615364]
30. Ngamkitidechakul C, Burke JM, O'Brien WJ, Twining SS. Maspin: synthesis by human cornea and regulation of in vitro stromal cell adhesion to extracellular matrix. *Invest Ophthalmol.Vis.Sci.* 2001; 42:3135–3141. [PubMed: 11726614]
31. Ngamkitidechakul C, Twining SS. Buffered non-fermenter system for lab-scale production of secreted recombinant His-tagged proteins in *Saccharomyces cerevisiae*. *Biotechniques.* 2002; 33:1296–1300. [PubMed: 12503315]
32. Ngamkitidechakul C, Warejcka DJ, Burke JM, O'Brien WJ, Twining SS. Sufficiency of the reactive site loop of maspin for induction of cell-matrix adhesion and inhibition of cell invasion. Conversion of ovalbumin to a maspin-like molecule. *J.Biol.Chem.* 2003; 278:31796–31806. [PubMed: 12799381]
33. Nykjaer A, Christensen EI, Vorum H, Hager H, Petersen CM, Roigaard H, Min HY, Vilhardt F, Moller LB, Kornfeld S, Gliemann J. Mannose 6-phosphate/insulin-like growth factor-II receptor targets the urokinase receptor to lysosomes via a novel binding interaction. *J.Cell Biol.* 1998; 141:815–828. [PubMed: 9566979]
34. Ottino P, Taheri F, Bazan HE. Platelet-activating factor induces the gene expression of TIMP-1, -2, and PAI-1: imbalance between the gene expression of MMP-9 and TIMP-1 and -2. *Exp.Eye Res.* 2002; 74:393–402. [PubMed: 12014920]
35. Petroll WM, Jester JV, Barry-Lane PA, Cavanagh HD. Effects of basic FGF and TGF beta 1 on F-actin and ZO-1 organization during cat endothelial wound healing. *Cornea.* 1996; 15:525–532. [PubMed: 8862930]
36. Ritchie H, Booth NA. Secretion of plasminogen activator inhibitor 2 by human peripheral blood monocytes occurs via an endoplasmic reticulum-golgi-independent pathway. *Exp.Cell Res.* 1998; 242:439–450. [PubMed: 9683531]
37. Seftor RE, Seftor EA, Sheng S, Pemberton PA, Sager R, Hendrix MJ. maspin suppresses the invasive phenotype of human breast carcinoma. *Cancer Res.* 1998; 58:5681–5685. [PubMed: 9865722]
38. Stramer BM, Zieske JD, Jung JC, Austin JS, Fini ME. Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes. *Invest Ophthalmol.Vis.Sci.* 2003; 44:4237–4246. [PubMed: 14507867]
39. Taylor JL, Casey MS, O'Brien WJ. Synergistic antiherpes virus activity of acyclovir and interferon in human corneal stromal cells. *Invest Ophthalmol.Vis.Sci.* 1989; 30:365–370. [PubMed: 2466806]
40. Teoh SS, Whisstock JC, Bird PI. Maspin (SERPINB5) is an obligate intracellular serpin. *J.Biol.Chem.* 2010; 285:10862–10869. [PubMed: 20123984]
41. Twining SS. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal.Biochem.* 1984; 143:30–34. [PubMed: 6442109]
42. Twining SS, Fukuchi T, Yue BY, Wilson PM, Zhou X, Loushin G. Alpha 2-macroglobulin is present in and synthesized by the cornea. *Invest Ophthalmol.Vis.Sci.* 1994; 35:3226–3233. [PubMed: 7519181]
43. Twining SS, Wilson PM, Ngamkitidechakul C. Extrahepatic synthesis of plasminogen in the human cornea is up-regulated by interleukins-1alpha and -1beta. *Biochem.J.* 1999; 339(Pt 3):705–712. [PubMed: 10215610]
44. Warejcka DJ, Vaughan KA, Bernstein AM, Twining SS. Differential conversion of plasminogen to angiostatin by human corneal cell populations. *Mol.Vis.* 2005; 11:859–868. [PubMed: 16270025]
45. Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell.* 1997; 91:439–442. [PubMed: 9390552]

46. West-Mays JA, Sadow PM, Tobin TW, Strissel KJ, Cintron C, Fini ME. Repair phenotype in corneal fibroblasts is controlled by an interleukin-1 alpha autocrine feedback loop. *Invest Ophthalmol. Vis. Sci.* 1997; 38:1367–1379. [PubMed: 9191600]
47. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* 1991; 5:2145–2154. [PubMed: 1850705]
48. Zhang G, Cai X, Lopez-Guisa JM, Collins SJ, Eddy AA. Mitogenic signaling of urokinase receptor-deficient kidney fibroblasts: actions of an alternative urokinase receptor and LDL receptor-related protein. *J.Am.Soc.Nephrol.* 2004; 15:2090–2102. [PubMed: 15284295]
49. Zhang G, Kernan KA, Thomas A, Collins S, Song Y, Li L, Zhu W, Leboeuf RC, Eddy AA. A novel signaling pathway: fibroblast nicotinic receptor alpha1 binds urokinase and promotes renal fibrosis. *J.Biol.Chem.* 2009; 284:29050–29064. [PubMed: 19690163]
50. Zhang M, Volpert O, Shi YH, Bouck N. Maspin is an angiogenesis inhibitor. *Nat.Med.* 2000; 6:196–199. [PubMed: 10655109]
51. Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, Rafidi K, Seftor E, Sager R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science.* 1994; 263:526–529. [PubMed: 8290962]

Figure 1.

Maspin increases tPA and uPA in fibroblast and myofibroblast conditioned media. Conditioned medium from cultured human corneal stromal fibroblasts (Fib) and myofibroblasts (Myo) were treated with control medium or medium containing 1 μ M maspin for different time periods. Samples of conditioned medium were normalized based on cellular protein levels and run on casein-plasminogen zymogram gels. The bands represent both pro and active forms of the plasminogen activators. No proteolytic bands were noted on control casein gels (no plasminogen added) for the corneal cells (data not shown). Similar results were observed for four additional donors.

Figure 2.

Maspin does not alter fibroblast and myofibroblast cell associated uPA or tPA levels.

Fibroblasts (**A**) and myofibroblasts (**B**) were treated with or without 1 μ M maspin. Equal amounts of solubilized cellular protein were separated on casein-plasminogen zymogram SDS-PAGE.

Figure 3.

Maspin increases secreted PAI-1 produced by fibroblasts and myofibroblasts. Human corneal stromal fibroblasts and myofibroblasts were treated with or without 1 μ M maspin and the conditioned medium was separated on SDS-PAGE gels. Samples were normalized based on cellular protein levels. The blots were probed with a mouse monoclonal antibody to PAI-1. Similar results were observed for five additional sets of cells.

Figure 4.

Maspin increases total plasminogen activation activity in fibroblast and myofibroblast conditioned medium. Human corneal stromal fibroblasts and myofibroblasts were cultured in the presence or absence of 1 μ M maspin. The conditioned medium was normalized for cellular proteins and the active plasminogen activators present assayed using the FITC-casein assay in the presence of plasminogen. Plasminogen activator activity was increased in medium conditioned by fibroblasts (**A**) or myofibroblasts (**B**) cultured in the presence of maspin. A two-tailed ANOVA demonstrated an overall difference in the presence and absence of maspin for both the fibroblasts and myofibroblasts. Individual comparisons using the Bonferroni post-test revealed significant differences at 8 and 24 hrs for both types of cell with and without maspin treatment ($p < 0.01$). This was confirmed in fibroblasts (**C**) and myofibroblasts (**D**) from 6 additional individual donors cultured for 24 hrs with the exception of one set of myofibroblasts. In the presence of maspin, plasminogen activation was significantly increased relative to cells cultured in the absence of maspin using the two-tailed-standard paired t-test ($p < 0.005$).

Figure 5.

Plasminogen activation in the presence of maspin is increased. Conditioned medium from corneal stromal fibroblasts and myofibroblast cultured in the presence and absence of maspin were incubated for an additional twenty-four hours in the presence or absence of plasminogen. Samples were normalized based on cellular protein levels. The medium was separated on casein zymogram gels. Pln = plasmin standard.

A **B** **Figure 6.**

Maspin does not significantly alter cell associated plasminogen activation or uPAR levels. (A) Human corneal fibroblasts and myofibroblasts were cultured in medium with or without 1 μ M maspin. After treatment the cells were washed and incubated with or without maspin in DMEM containing FITC-casein and plasminogen to determine plasminogen activator activity. Graph is representative of three individual experiments using cells from different donors. $n=3$ for points. No differences were observed between the curves for fibroblasts or myofibroblasts in the presence of absence of maspin using two-way ANOVA. (B) Lysates of stromal fibroblasts, cultured with and without 1 μ M maspin, were normalized for protein and separated by SDS-PAGE. The proteins were transblotted to nitrocellulose and the membranes were probed for uPAR.

Figure 7.

Maspin does not alter mRNA levels for tPA nor uPA in corneal stromal fibroblasts and myofibroblasts. Fibroblasts and myofibroblasts from three donors were treated with or without 1 μ M maspin for 1 hour. The mRNA was isolated and used in a SYBR Green QPCR using specific primers for tPA (**A**) and uPA (**B**). All samples were normalized to GAPDH mRNA levels and expressed relative to the control fibroblast mRNA levels as 1 relative unit. $n = 3$ independent experiments using cells from different donors. Error bars = std error of the mean. No significant differences were observed using the Student's *t*-test between mRNA levels for tPA or uPA in fibroblasts or myofibroblasts cultured in the presence or absence of maspin.

Figure 8.

Maspin inhibits turnover of uPA in myofibroblasts but not uPA and tPA in fibroblasts. Cultures of fibroblasts (**A – F**) and myofibroblasts (**G–J**) were treated 16 hr with/or without 10 μ g/ml cycloheximide, then treated with/or without maspin for 1 to 6 hrs. Conditioned medium from cells were separated on zymogram SDS-PAGE. The gels (**A, D, G** and **I**) were analyzed using ImageJ and the results plotted (**B, C, E, F, H, J**). Shown are representative gels of three independent experiments.

Figure 9.

Maspin stimulates production of angiostatin in fibroblast and myofibroblast conditioned medium. Corneal stromal fibroblasts (Fib) and myofibroblasts (Myo) were treated with or without maspin for 24 hours. Conditioned medium from these cells were incubated for 72 hours with plasminogen, then run on SDS-PAGE, transblotted to nitrocellulose membrane and probed with antibodies to kringle 1–3 of plasminogen. Bands with molecular weights in the range of 37–48 kDa represent angiostatin-like molecules.