Maspin: Synthesis by Human Cornea and Regulation of In Vitro Stromal Cell Adhesion to Extracellular Matrix

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Purpose. Maspin, a tumor-suppressor protein that regulates cell migration, invasion, and adhesion, is synthesized by many normal epithelial cells, but downregulated in invasive epithelial tumor cells. The purpose of this study was to determine whether cells in the normal human cornea express maspin and whether maspin affects corneal stromal cell adhesion to extracellular matrix molecules.

Methods. Maspin expression was analyzed by immunodot blot, Western blot, and RT-PCR analyses in cells obtained directly from human corneas in situ. Maspin protein and mRNA were also studied in primary and passaged cultures of corneal stromal cells using Western blot analysis, RT-PCR, and immunofluorescence microscopy. Maspin cDNA was cloned and sequenced from human corneal epithelial cells and expressed in a yeast system. The recombinant maspin was used to study attachment of cultured human corneal stromal cells to extracellular matrices.

Results. Maspin mRNA and micromolar amounts of the protein were found in all three layers of the human cornea in situ, including the stroma. Maspin was also detected in primary and first-passage corneal stromal cells, but its expression was downregulated in subsequent passages. Late-passage stromal cells, which did not produce maspin, responded to exogenous recombinant maspin as measured by increased cell adhesion not only to fibronectin, similar to mammary gland tumor epithelial cells, but also to type I collagen, type IV collagen, and laminin.

Conclusions. The corneal stromal cell is the first nonepithelial cell type shown to synthesize maspin. Loss of maspin expression in late-passage corneal stromal cells in culture and their biological response to exogenous maspin suggests a role for maspin on the stromal cells in the cornea. Maspin may function within the cornea to regulate cell adhesion to extracellular matrix molecules and perhaps to regulate the migration of activated fibroblasts during corneal stromal wound healing. (*Invest Ophthalmol Vis Sci.* 2001;42:3135–3141)

Maspin is a member of the serine proteinase inhibitor (SERPIN) superfamily of proteins that includes α-1 antitrypsin, plasminogen activator inhibitor, pigment epithelial–derived factor, and ovalbumin.[1] Despite its similarity to other SERPINs, it is unclear whether maspin functions as a proteinase inhibitor.[2,3] Maspin is expressed by a variety of normal epithelial cells of mammalian organs including mammary gland, prostate, skin, stomach, and thymus.[4–6] This molecule is present both within cells and associated with the extracellular matrix (ECM) of these tissues. However, in mammalian carcinoma cell lines such as the mammary gland epithelial tumor cell line MDA-MB-231, maspin expression is downregulated, but the gene is not mutated.[7]

Maspin is a tumor-suppressor protein that inhibits epithelial tumor cell motility and invasion in vitro and suppresses tumor metastasis in nude mice.[4,8] The inhibition of cell motility appears to result from maspin's ability to enhance tumor cell attachment to the ECM molecule fibronectin.[9] Maspin is also an inhibitor of angiogenesis, as indicated by its ability to inhibit bFGF-induced proliferation and migration of microvascular endothelial cells in vitro and to block neovascularization in vivo in the rat cornea pocket model.[10] Therefore, maspin inhibits tumorigenesis, not only by acting directly on the tumor cells, but also by inhibiting the angiogenesis required for tumor growth.

We hypothesized that maspin is present in the cornea, a transparent tissue which requires the absence of blood vessels and in which tumors are rarely found.[11] As a product of many epithelial cell types, we expected to find that maspin is synthesized in the corneal epithelium and/or in the endothelium. In this study, these corneal cells indeed synthesized maspin. Unexpectedly, maspin was also expressed by the corneal stroma, which consists of nonepithelial cells (keratocytes) surrounded by a largely collagenous matrix. Because maspin is known to regulate the attachment of tumor cells to the ECM molecule fibronectin, we considered that maspin may play a similar regulatory role on ECM adhesion of corneal stromal cells during wound healing. We studied corneal stromal cells, not only for their ability to synthesize maspin, but also to determine whether stromal cells treated with maspin exhibit altered adhesion to ECM molecules found in normal or wounded corneas.

Methods

Cell Cultures

Human mammary gland carcinoma MDA-MB-231 cells from American Type Culture Collection (Manassas, VA) were maintained at 37°C without CO_2 in Leibovitz's L-15 medium (Life Technologies, Baltimore, MD) with 2 mM L-glutamine and 10% fetal bovine serum (Sigma, St. Louis, MO).

Human corneal stromal cells were recovered by collagenase digestion of human corneas obtained from the Wisconsin Lion Eye Bank (Milwaukee, WI), as previously described by Taylor et al.[12] The cultured corneal stromal cells were grown in flasks (Costar, Cambridge, MA) containing high-glucose DMEM with L-glutamine (Life Technologies) supplemented with 5% FBS defined (Hyclone, Logan, UT), 0.1% Mito$^+$ serum extender (Collaborative Research, Bedford, MA), and 10 μg/ml ciprofloxacin (Bayer, Kankakee, IL) at 34°C. Cells were split at a ratio of 1:2 or 1:3.

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Preparation of Protein Extract and Total RNA

Human corneas (obtained from the Lions Eye Bank of Wisconsin) were dissected and the epithelial, stromal, and endothelial layers dissociated. Protein extracts from the tissues were prepared in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.15 M NaCl at 4°C. The epithelial and endothelial layers were homogenized using a ground-glass tissue grinder, and the stromal proteins extracted using a homogenizer (Polytron; Brinkmann, Westbury, NY). To extract total RNA, the corneal epithelial and endothelial layers were scraped and directly transferred into extraction reagent (TRI Reagent; Molecular Research Center, Cincinnati, OH). The stromal layer was ground in a percussion chamber cooled with liquid nitrogen and then transferred into extraction reagent. To prepare total RNA and total protein from the cultured corneal stromal cells, extraction reagent was directly added to the culture flasks. Isolation of total RNA and protein was then performed as described in the manufacturer's protocol.

Western Blot Analysis

Supernatant fractions of each extract were electrophoresed on 10% SDS-PAGE gels under reducing conditions, and electrotransferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ), as described elsewhere.[13] Maspin was detected in the epithelial and endothelial extracts by rabbit anti-human maspin IgG antibodies affinity purified on a maspin column and shown to be specific for maspin.[6] The maspin antibodies were generous gifts from Phillip A. Pemberton (LXR Biotechnology, Richmond, CA). Nonspecific rabbit IgG (Bio-Rad, Hercules, CA) was used as a control on duplicate blots. Affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP; Bio-Rad) was used as the secondary antibody. For the stromal extract, a mouse monoclonal anti-human maspin antibody (BD PharMingen, San Diego, CA) was used as a primary antibody and HRP-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) as the secondary antibody. To detect corneal epithelium–specific cytokeratin 3, a mouse monoclonal anti-human cytokeratin-3 antibody (Research Diagnostics, Inc., Flanders, NJ) was used as the primary antibody and goat anti-mouse IgG conjugated with HRP (Pierce) as the secondary antibody. For detection of recombinant FLAG/His-tagged maspin, a nickel-conjugated HRP (INDIA HisProbe-HRP; Pierce) was used. The HRP-labeled bands were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). Unless otherwise indicated, all results were confirmed in at least two independent experiments on tissue extracts or cultured cells from different donors.

Immunodot Blot Assay

The total amount of protein extracted from the corneal epithelial, stromal, and endothelial layers was determined by the Lowry assay using bovine serum albumin (BSA; Sigma) as a standard protein.[14] The supernatant fraction of corneal total protein extracts and bacterial recombinant maspin standards were loaded onto a nitrocellulose membrane in a 96-well dot blot apparatus (Schleicher & Schuell, Keene, NH). The dot blot was processed in the same manner as described for the Western blot analysis, using rabbit polyclonal anti-human maspin antibodies for the epithelial and endothelial extracts and the mouse monoclonal anti-human maspin antibody for the stromal extracts. Nonspecific binding was determined on duplicate blots using nonspecific rabbit or mouse IgG plus the secondary antibodies or the secondary antibodies alone as described for the Western blot analysis. The data were analyzed using an ELISA plate reader (EL380 Microplate Reader; Bio-Tek Instruments, Winooski, VT) with a 530-nm filter. Each sample was assayed in triplicate. Linear regression analyses of standard maspin preparations were used to determine the maspin concentration in each sample. The values were averaged and the SD was determined.

Reverse Transcription–Polymerase Chain Reaction

Maspin cDNA was synthesized from 0.2 to 1 μg total RNA from individual corneal layers or stromal cells using random hexamers and murine leukemia virus (MuLV) reverse transcriptase (PE Biosystems, Foster City, CA) at 42°C for 15 minutes and then amplified by the polymerase chain reaction (PCR) using *Taq* DNA polymerase (Ampli-*Taq* Gold; PE Biosystems) according to the manufacturer's protocol. All oligonucleotide primers were from (Gibco BRL-Life Technologies). A 468-bp PCR fragment was amplified using primers specific to the reactive site loop (RSL) region of maspin (5′-AGGATGTGGAGGATGAG-3′ and 5′-ACAGAAAAGTCAGGGAGG-3′). A three-step temperature cycling for PCR was 1 minute at 95°C for denaturation, 1 minute at 55°C for annealing, and 1 minute at 72°C for extension. A 1.2-kb fragment containing the maspin open reading frame (ORF) was generated using the PCR primers 5′-CGGAGATCTGCGGCCGCAATGGATGCCCTGC-3′ and 5′-CCGCTCGAGGAATTCA-CATGTGCTATGCCACT-3′. The PCR products were cloned into a vector (pGEM-T Easy; Promega, Madison, WI), and then manually sequenced (T7 Sequenase V 2.0 kit; Amersham Pharmacia Biotech). The sequences were compared with the published human maspin sequence from GenBank using the GCG software program (GenBank is provide in the public domain by the National Center for Biotechnology, Bethesda, MD, and is available at http//:www.ncbi.nlm.nih.gov/genbank).

Immunofluorescence Microscopy

Corneal stromal cells were cultured in eight chamber slides (Nalge Nunc International, Rochester, NY). The cells were fixed for 15 minutes with cold 3% paraformaldehyde, then permeabilized by incubation for 5 minutes at room temperature in 0.5% Triton X-100 in phosphate-buffered saline (PBS). Fixed monolayers were incubated with primary rabbit anti-maspin antibodies for 1 hour at room temperature, followed by three rinses in blocking buffer (10 mg/ml BSA in PBS) and a 1-hour incubation in tetrarhodamine isothiocyanate (TRITC)–conjugated donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Next, these cells were costained for F-actin with FITC-phalloidin (Sigma) for 30 minutes at room temperature. After staining, coverslips were mounted with antifade reagent (FluoroGuard; Bio-Rad), and specimens were examined and photographed with a fluorescence microscope. Control slides were stained using nonimmune rabbit IgG. All results were confirmed in at least two independent experiments on samples from different donors.

Yeast Vector Construction and Expression of Recombinant Maspin

Recombinant maspin was produced in yeast (FLAG expression system; Sigma) with modifications to the vector. Six histidine residues were incorporated into the YEpFLAG-1 vector downstream of the FLAG peptide sequence by overlapping PCR (Fig. 1A). The maspin open reading frame (ORF) was amplified from the pGEM vector by PCR using DNA polymerase (*Pfu* Turbo polymerase; Stratagene, La Jolla, CA). To subclone into the YEpFLAG/His-1 vector, *Eco*RI and *Bgl*II sites were incorporated into both ends using primers 5′-CGGAATTCATGGATGCCCTGCAACTA-3′ and 5′-GCAGATCTTTAAGGAGAACAGAATTT-3′. The maspin ORF was subcloned into the YEpFLAG/His-1 vector in which the FLAG/His was tagged to N-terminus of maspin ORF (Fig. 1A). The YEpFLAG/His-Maspin construct was transformed into the *Saccharomyces cerevisiae* protease-deficient yeast strain BJ3505 (Sigma) using a lithium acetate transformation method, as described in the manufacturer's expression system manual. Yeast recombinant FLAG/His maspin was expressed in YPHSM medium (1% glucose, 3% glycerol, 1% yeast extract, 8% peptone, and 20 mM $CaCl_2$). The cultures were grown on a rotary platform at 175 rpm at 30°C for 72 hours.

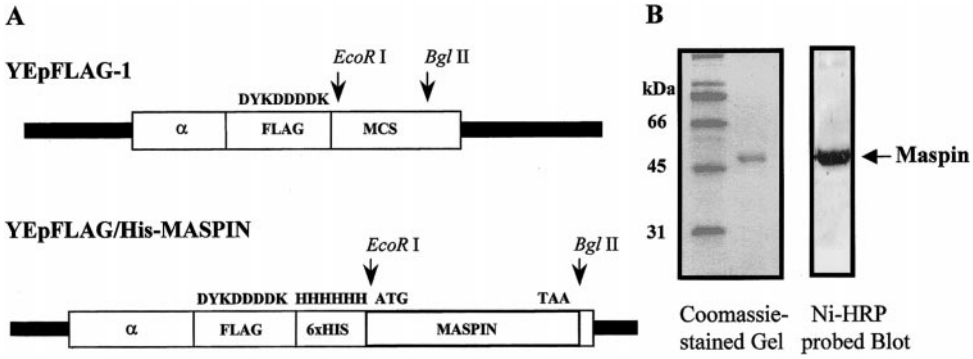

FIGURE 1. Expression and purification of biologically active recombinant FLAG/His-maspin in yeast. (**A**) A six-histidine tag was incorporated after the FLAG sequence into the original YEpFLAG-1 expression vector by overlapping insertion PCR. The maspin ORF cDNA cloned from human corneal epithelium was then inserted between the *Eco*RI and the *Bgl*II sites of the multiple cloning site (MCS). This vector was used for expression maspin in the yeast *S. cerevisiae* protease-deficient strain BJ3505. The recombinant maspin was secreted into the YPHSM yeast medium and purified using a cobalt affinity resin. (**B**) Purified yeast recombinant FLAG/His tagged maspin was separated on a 10% polyacrylamide gel by SDS-PAGE and detected by Coomassie brilliant blue staining (*left*) and on an electroblot using a nickel-conjugated HRP and the ECL system (*right*).

Purification of the Recombinant Flag/His-Tagged Maspin from Yeast

Because of the presence of an alpha factor leader sequence, the recombinant protein was secreted into the yeast culture medium. The cells were removed by centrifugation at 4500*g*, and the yeast culture supernatant was concentrated to half the volume by ultrafiltration, using a 30-kDa cutoff membrane (BioMax Millipore Corp., Bedford, MA). The protein was buffer exchanged into a binding buffer (50 mM phosphate buffer [(pH. 8.0]) containing 300 mM NaCl) by diluting the sample 10× with buffer. The recombinant FLAG/His maspin in the supernatant was purified using cobalt affinity resin (Talon Superflow; Clontech, Palo Alto, CA). Large-scale batch purification was performed according to the manufacturer's protocol. The eluted fractions containing recombinant maspin were pooled, and the imidazole removed by repeated dilution with PBS and concentrated using a 30-kDa cutoff centrifugal filter (Ultafree; Millipore).

The purified recombinant maspin was subjected to SDS-PAGE under reducing conditions and detected by Coomassie blue staining (Fig. 1B) or after electroblot transfer to nitrocellulose and then detected by the nickel-conjugated HRP (INDIA HisProbe-HRP; Pierce; Fig. 1B). The hexahistidine tag, the FLAG peptide composed of eight amino acids, and glycosylation account for the extra 8-kDa over the native size of 42 kDa. Approximately 1 mg purified yeast recombinant maspin was produced per liter of yeast culture medium.

The biologic activity of recombinant maspin was tested on mammary gland carcinoma MDA-MB-231 cells using a cell–fibronectin adhesion assay, as described in the following section. The recombinant protein was biologically active, in that it increased the tumor cell attachment to fibronectin, similar to the recombinant bacterial maspin obtained from Phillip A. Pemberton (data not shown).

Cell–ECM Protein Adhesion Assay

Cell adhesion assays were conducted using ECM-coated plates (CytoMatrix; Chemicon International, Temecula, CA) according to the manufacturer's instructions.

MDA-MB-231 cells and corneal stromal cells were cultured in MEM plus 2% lactalbumin (Sigma) and the human stromal culture medium without FBS, respectively. Subconfluent cells were pretreated overnight with 1 μM yeast recombinant maspin. As a negative control, ovalbumin (grade VII, essentially free of S-ovalbumin; Sigma), a SERPIN very homologous to maspin, was included in the assay. The cells were harvested using enzyme-free, Hanks'-based, cell dissociation buffer (Life Technologies). They were washed with PBS, resuspended in medium, and counted using a hemocytometer. Approximately 2×10^4 cells were plated on cell adhesion strips (CytoMatrix; Chemicon International), precoated with ECM proteins or with BSA as a negative control. After incubation at 37°C for 1 hour, the nonadherent cells were removed by gently washing with PBS containing 1 mg/ml $CaCl_2$ and 1 mg/ml $MgCl_2$. The adherent cells were stained with 0.2% crystal violet in 10% ethanol. The excess stain was removed by gently washing three times with PBS. The attached cells were then solubilized with a 1:1 mixture of 0.1 M NaH_2PO_4 (pH 4.5) and 50% ethanol. Cell attachment was determined by measurement of dye color at 550 nm on an ELISA microplate reader. Each experiment contained at least triplicate samples for each condition. Four independent experiments were performed with cells from different donors.

Statistical analysis was performed (Sigma Stat software; SPSS Inc., Chicago, IL) using a one-way analysis of variance for overall differences among control, maspin, and ovalbumin treatment groups. The significance of differences between mean values of optical density at 550 nm was determined using the Student-Newman-Keuls method, with $P < 0.05$ indicating significance.

RESULTS

Expression of Maspin in All Three Layers of the Human Cornea

Maspin was found in the epithelium of the human cornea similar to the epithelium of mammary gland, prostate, and skin.[5,6,15] In addition to the epithelium, maspin was also specifically detected in the stromal and endothelial layers of the cornea (Fig. 2A, 2B). Absence of the epithelial marker protein cytokeratin 3 in the stromal extract was used to assure that maspin from the epithelium did not contaminate the stromal samples (not shown). Quantification of maspin in the three layers of human cornea by immunodot blot assay using maspin-specific antibodies (Table 1) showed that the highest total amount of maspin was found in the stroma, although the concentration per milligram of extracted protein was similar in the epithelial and stromal extracts. The lowest amount was detected in the endothelial layer.

RT-PCR analysis demonstrated that cells of the epithelium, the endothelium, and, notably, the stroma synthesize maspin (Figs. 2C–E). Using primers specific for the maspin RSL region, an ~465-bp RSL RT-PCR product was amplified from total RNA extracted from all corneal layers. Control analyses without reverse transcriptase confirmed the absence of contaminating DNA.

Total RNA from the epithelial and stromal layers was further analyzed by synthesizing cDNA of the entire 1.2-kb maspin ORF (Fig. 2E). Digestion of the 1.2-kb PCR products with restriction enzymes *Bam*HI, *Dde*I, *HinD*III, *Alu*I, and *Nco*I yielded digestion patterns predicted from the sequences of human maspin gene (not shown). DNA sequencing confirmed the identity of the gene. Compared with the human mammary gland maspin sequence (GenBank, U04313), nucleotide substi-

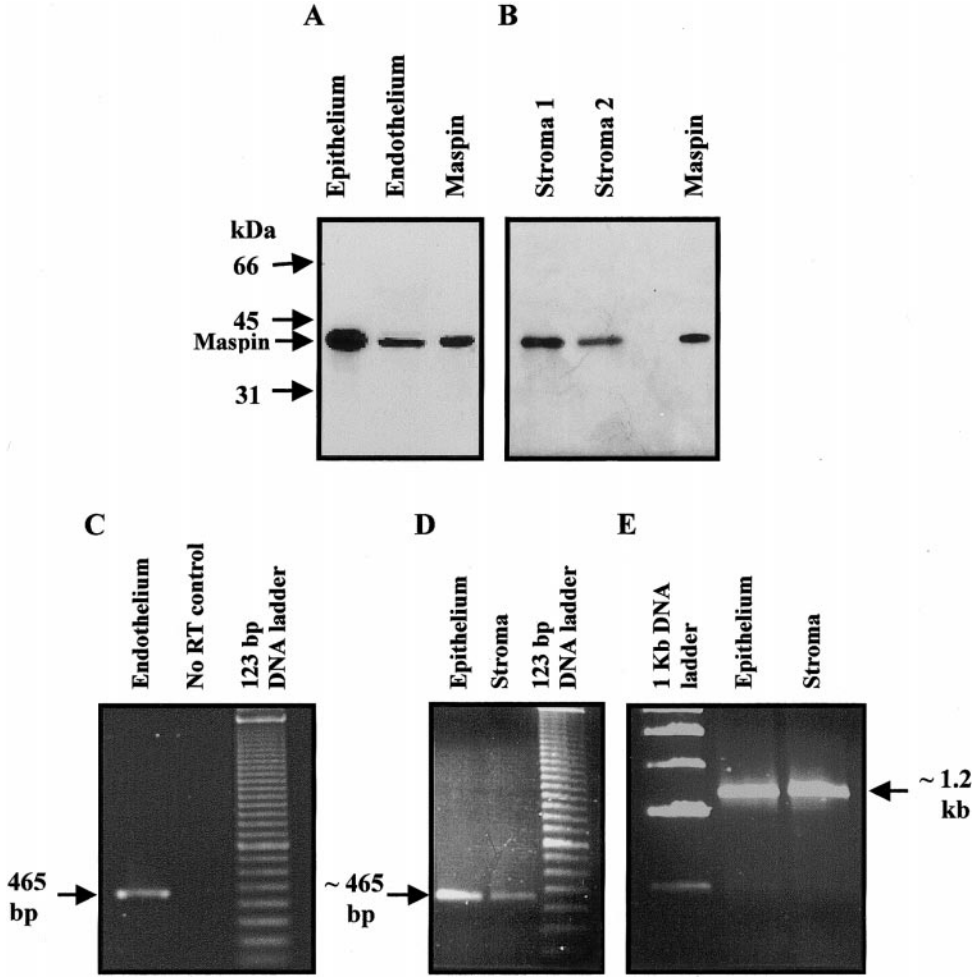

FIGURE 2. Identification of maspin protein and mRNA in the three layers of human cornea. (**A**) Immunoblot analysis of maspin isolated from corneal epithelium and endothelium. Protein extracts from each layer were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membrane. The membrane was probed with affinity-purified rabbit polyclonal anti-human maspin antibodies and secondary peroxidase-conjugated goat anti-rabbit IgG antibodies, followed by ECL detection. (**B**) Immunoblot analysis of maspin isolated from the stroma of two corneas from different individuals (*lanes 1*, *2*) probed with a mouse monoclonal anti-human maspin antibody and secondary HRP-conjugated goat anti-mouse IgG antibodies, followed by ECL detection. Longer exposures of these blots and comparison with control blots probed with nonspecific rabbit or mouse IgG followed by the respective secondary antibodies confirmed the specificity of these bands (not shown). (**C**) RT-PCR analysis of maspin mRNA from human corneal endothelium. Total RNA (~200 ng) was reversed transcribed using murine leukemia virus (MuLV) reverse transcriptase and random hexamers as primers. The ~465-bp cDNA was PCR amplified using *Taq* DNA polymerase with primers specific to the unique maspin RSL and the 3′ end of the ORF. Control reactions without reverse transcriptase produced no PCR products. (**D**) RT-PCR analysis of the maspin from epithelium and stroma. The ~465-bp cDNA was RT-PCR amplified using a specific maspin RSL primer and a primer to the 3′ end of the ORF. (**E**) The ~1.2-kb PCR products of maspin ORF were amplified using a primer set specific to both ends of the maspin ORF.

tutions were found at positions 196 (A → G) and 243 (T → C). The first substitution caused a missense mutation that encodes Val instead of Ile at amino acid residue 66. This Val substitution was previously reported in the maspin sequences for mouse mammary gland[15] (GenBank, U54705), rat prostate[5](GenBank: U58857), and human prostate.[5] The second substitution was a conservative change that encodes the same amino acid, a serine at residue 81.

Expression of Maspin in Corneal Stromal Cells in Culture

To further confirm the unexpected finding that corneal stromal cells express maspin, stromal cell cultures were established and analyzed by RT-PCR and Western blot analysis for maspin message and protein, respectively. Total RNA and protein extracts were prepared from cells of various passages cultured from the same tissue donor. As shown in Figure 3A, the ~465-bp maspin-specific RT-PCR products were amplified from the total RNA extracts from both primary and first-passage cells. However, maspin expression declined to undetectable levels after further culture propagation (Fig. 3A, passages 2 to 3). Similar to the RT-PCR result, Western blot analysis showed maspin protein was present in both primary and first-passage cultures, but was absent in late-passage cells (Fig. 3B). As shown in Figure 3C, the two higher molecular weight bands were clearly nonspecific, resulting from the secondary HRP-conjugated goat anti-rabbit IgG antibodies.

TABLE 1. Levels of Maspin in Normal Human Cornea

	n	Maspin/Cornea (μg)*	Maspin/mg Protein† (μg)
Epithelium	5	2.6 ± 0.9‡	9.5 ± 1.6
Stroma	5	9.8 ± 3.5	7.7 ± 1.7
Endothelium	2	0.036 (0.020, 0.051)§	0.43 (0.24, 0.61)

* Maspin concentrations were determined by immunodot blot analysis using the rabbit polyclonal anti-maspin antibodies for the epithelium and the endothelium and the mouse monoclonal anti-maspin antibody for the stroma as given in Figures 2A and 2B. Maspin standard curves were linear. Control blots using nonimmune rabbit or mouse IgG were blank.

† Extracted total protein using bovine serum albumin for the protein standard curve.

‡ SD.

§ Average value of two pools of five corneas each (values for each pool).

Immunofluorescence microscopy showed variable amounts of maspin in primary and first-passage stromal cells but not in later passage cells (Fig. 4). It distributed in a diffuse-punctate pattern, especially in the nuclear/perinuclear regions. This staining was specific, as illustrated in a first-passage culture. In contrast to the HRP-conjugated goat anti-rabbit IgG antibodies used for the Western blot analysis, the TRITC-conjugated donkey anti-rabbit IgG antibodies did not react with the primary-through-late–passage cells.

Effect of Maspin on Corneal Stromal Cell Adhesion to ECM

Loss of maspin expression with passage of cultured corneal stromal cells resembles the downregulation of maspin observed when normal mammary gland epithelial cells are converted to carcinoma cells.[4] Pretreatment of tumor cells, which do not express maspin, with exogenous maspin increases cell adhesion to fibronectin.[9] To determine whether later passage corneal stromal cells that do not express maspin respond to exogenous maspin, fourth- or fifth-passage stromal cells were pretreated with yeast recombinant maspin. Their attachment to ECM molecules was compared with that of control cells without treatment and cells pretreated with the related SERPIN ovalbumin. Not only was fibronectin tested, but also type I collagen, type IV collagen, and laminin, ECM molecules to which stromal cells might be exposed in the normal cornea and/or during corneal wound healing.

As shown in Figure 5, pretreatment with exogenous maspin increased attachment of stromal cells to fibronectin, similar to the effect of maspin on carcinoma cells.[9] In contrast to maspin-treated carcinoma cells, maspin also increased stromal cell adhesion to type I collagen, type IV collagen, and laminin. Cell attachment to type I collagen was increased by approximately 70%, to laminin by 50%, and to type IV collagen or fibronectin by 30%. Maspin did not increase cell attachment to the non-biologic substrate BSA, and the homologous SERPIN ovalbumin had no significant effect on cell adhesion.

DISCUSSION

Human corneal cells in the epithelium, endothelium, and stroma express maspin. It is not surprising to find maspin in the stratified epithelium and monolayer endothelium of the cornea, because maspin has been detected both in multilayered skin epithelium and monolayer epithelia of mammary gland and stomach.[6] However, it is unusual to find maspin expression in the corneal stroma, which is the first nonepithelial tissue identified in which maspin is expressed. As shown by RT-PCR, using stromal tissue extracts and early passage stromal cells, maspin is a product of the stromal cells. Maspin synthesis by corneal stromal cells may be related to their unusual embryonic origin. Connective tissue cells are usually derived from mesoderm, whereas the corneal stroma arises from neural crest, a neuroectodermal tissue that is originally epithelial.[16,17] Further, unlike other connective tissues, the corneal stroma must be transparent to pass light to the retina and must remain avascular. Synthesis of anti-angiogenic proteins such as maspin may therefore be peculiarly important for function of the corneal stroma. Not only do the stromal cells produce maspin, but the adjacent epithelium and endothelium also synthesize maspin, which may function in the corneal stroma. Maspin is known to be secreted by skin keratinocytes in culture[18] and has been detected in the ECMs of other tissues.[4,6] Whether maspin found in the stroma originates only from stromal cells or from both stromal and adjacent cells is not known. Maspin (42 kDa) is present in the corneal stroma (volume, ~0.1 ml) in the micromolar range (~2.5 μM); a level sufficient to be physiologically significant, because the concentration of maspin required for biologic activity is in the range of 0.3 to 1 μM.[9,10,19]

Although maspin is synthesized by corneal keratocytes in situ and by primary and first-passage corneal stromal cells in culture, its expression declines to undetectable levels in later passage cells. Other biosynthetic products also exhibit

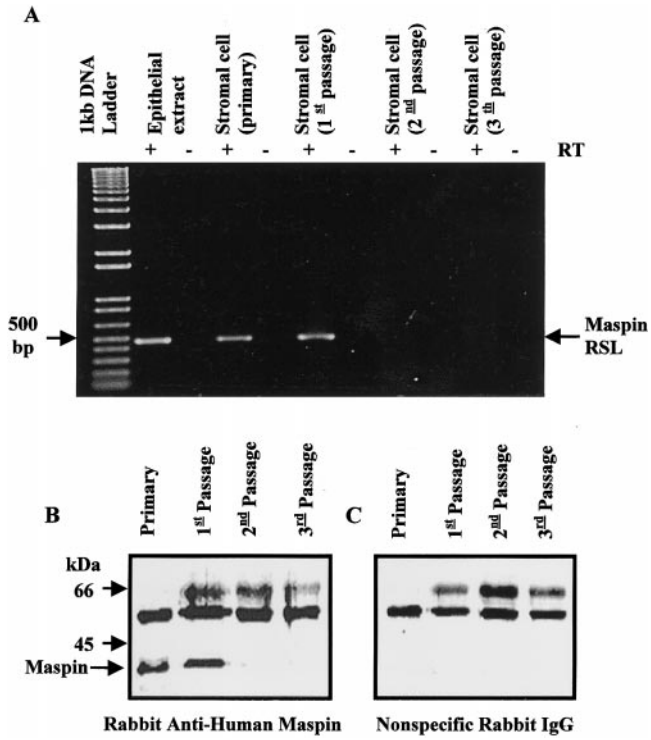

FIGURE 3. Expression of maspin in cultured human corneal stromal cells. Corneal stromal cells from the same tissue donor at different passages were analyzed for maspin transcript by RT-PCR (**A**) and Western blot (**B**). Total RNA and protein extracts were prepared using TRI Reagent. (**A**) Total RNA extracts from the same cultured cells were amplified by RT-PCR, using a specific primer to maspin RSL and the 3′ end of the ORF. Control reactions without reverse transcriptase (−RT) produced no PCR products. (**B**, **C**) Protein extracts (~2 μg) were loaded on SDS-PAGE gels, and electrotransferred onto nitrocellulose membranes. The membranes were probed with rabbit anti-human maspin antibodies followed by a secondary HRP-conjugated goat anti-rabbit IgG (**B**). A parallel blot was incubated only with the secondary antibodies (**C**). The bands were visualized, using an ECL system. Similar observations for both maspin mRNA and protein were obtained for a second set of cells from another donor.

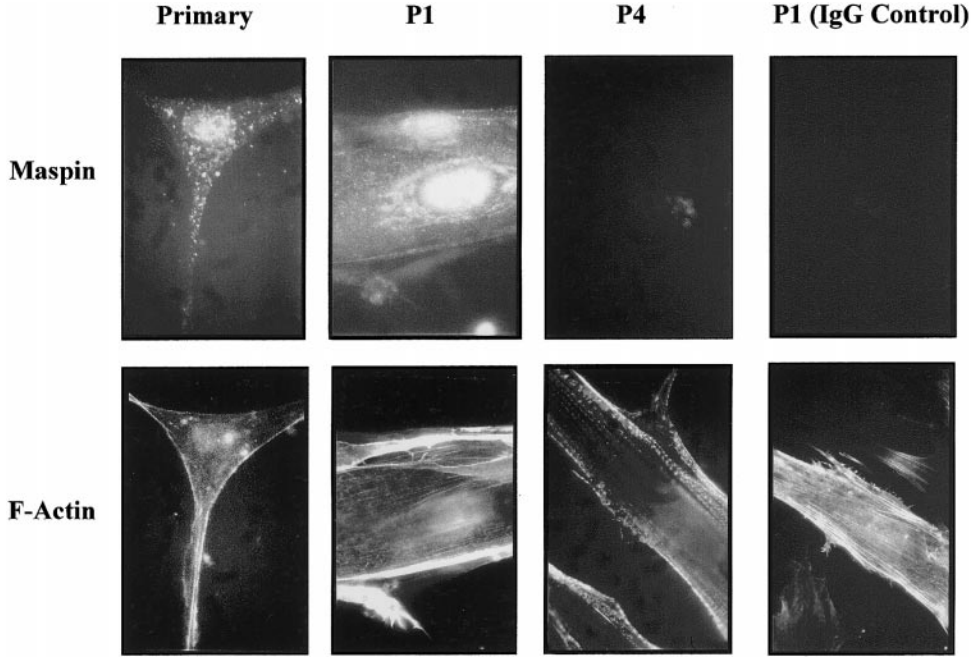

FIGURE 4. Immunofluorescence photomicrograph of maspin in cultured human corneal stromal cells. Primary, first-passage (P1), and fourth-passage (P4) human corneal stromal cells were probed with rabbit polyclonal anti-maspin antibodies (*top*) or nonimmune rabbit IgG control (IgG Control, P1) followed by TRITC-conjugated donkey anti-rabbit IgG antibodies. These cells were then colabeled with FITC-phalloidin to visualize the actin cytoskeleton (*bottom*). Original magnification, ×100.

changes in expression when human corneal stromal cells are propagated in vitro. For example, the synthesis of keratan sulfate proteoglycans dramatically decreases, and the production of the chondroitin-dermatan sulfate proteoglycan decorin and of basement membrane components laminin and perlecan increases in cultured cells.[20] Later passage cultured stromal cells have protein expression patterns that are similar to those of cells in wounded corneal stroma. Gene expression of molecules such as matrix metalloproteinases is induced both in cultured stromal cells and wounded corneal stroma.[21,22] These characteristics suggest that cultured corneal stromal cells resemble the activated

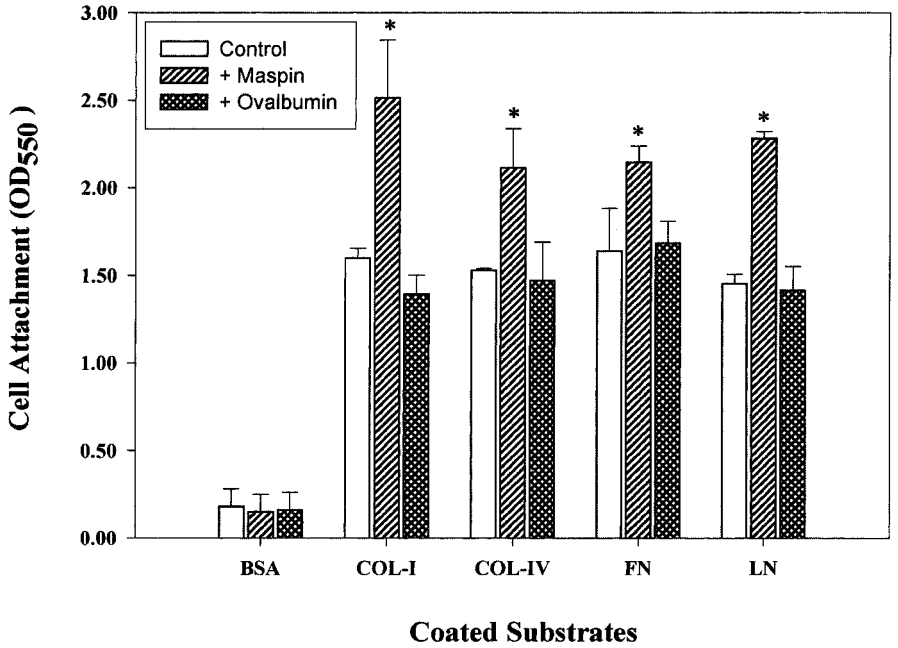

FIGURE 5. The effects of rFLAG/His-maspin on the adhesion of cultured human corneal stromal cells to various ECM molecules. After 24-hour treatment with 1 μM yeast recombinant maspin, fifth-passage corneal stromal cells ($\sim 2 \times 10^4$ cells) were replated on wells precoated with BSA or ECM components; type I collagen, type IV collagen, fibronectin, and laminin for 1 hour. Adherent cells were stained with 0.2% crystal violet. The background control cells were not treated with maspin, and the negative control cells were treated with ovalbumin. Error bars, SD. Overall significant differences between groups were determined by the one-way ANOVA. The Student-Newman-Keuls method was used to determine significant differences between individual samples. No statistical differences in adhesion to ECM components were noted between background control and negative control cells. *Significantly more maspin-treated cells adhered to the four ECM molecules than control and ovalbumin-treated cells at the levels of $P < 0.05$.

fibroblasts in wounded cornea, and cultured cells are often used as a model of the wound fibroblasts.[23,24] The similarity between stromal cells in culture and fibroblasts in corneal wound raises the possibility that wound fibroblasts may, as do late-stage cultured cells, lose maspin expression.

Downregulation of maspin expression in corneal stromal cells after culturing is reminiscent of the downregulation that occurs in mammary gland epithelial cells after conversion into carcinoma cells.[4] Both carcinoma cells[9] and late-passage corneal stromal cells (shown herein) respond to exogenous maspin by increased cell adhesion to ECM. In carcinoma cells, increased cell adhesion to fibronectin, which appears to be mediated by upregulation of the fibronectin receptor $\alpha 5$ integrin,[9] results in decreased tumor cell migration. Maspin may similarly regulate corneal stromal cell migration by increasing cell adhesion to fibronectin.

In contrast to mammary gland tumor cells, maspin also increased corneal stromal cell adhesion to type I collagen, type IV collagen, and laminin. These ECM proteins, together with fibronectin,[25] are present in both the normal[26] and the wounded corneal stroma,[27,28] raising the possibility that maspin plays a regulatory role in stromal wound healing. A possible sequence of events in the wounded stroma is that the conversion of cells to wound fibroblasts results in downregulation of maspin expression, lower maspin levels in the cornea, and therefore a permissive environment for fibroblast migration. As corneal wound healing proceeds and maspin levels in the stroma are restored (perhaps maspin originates from the adjacent healing epithelium), wound fibroblasts may again become stationary due to maspin-induced increases in their adhesion to ECM molecules. Whether maspin regulates stromal wound healing as suggested remains to be determined.

Maspin may also have other functions in the corneal stroma, such as preventing tumor invasion and maintaining avascularity, activities that are consistent with its documented tumor suppressor[4] and antiangiogenic properties.[10] Although the functions of maspin in the cornea are currently speculative, the data presented herein indicate that the protein is found throughout the cornea, including in the stroma where cells theoretically both produce and respond to maspin.

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