Elucidating the Function of Secreted Maspin: Inhibiting Cathepsin D–Mediated Matrix Degradation

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Abstract

Cellular interaction with the extracellular milieu plays a significant role in normal biological and pathologic processes. Excessive degradation of basement membrane matrix by proteolytic enzymes is a hallmark of tumor invasion and metastasis, and aspartyl proteinase cathepsin D is implicated as a major contributor to this process. Maspin, a noninhibitory serpin, plays an important role in mammary gland development and remodeling. Expression of Maspin is decreased in primary tumors and lost in metastatic lesions. Maspin is mostly cytoplasmic and is partially secreted; however, the fate and function of secreted Maspin has remained mostly unexplored. We hypothesized that secreted Maspin is incorporated into the matrix deposited by normal mammary epithelial cells and thus could play a critical role in cathepsin D–mediated matrix degradation and remodeling of mammary tissue. In the absence of Maspin, as is the case with most cancer cells, matrix degradation proceeds unrestricted, thus facilitating the progression to metastasis. To test this, we employed an *in vitro* model where gels containing both types I and IV collagen were preconditioned with normal mammary epithelial cells to allow the incorporation of secreted Maspin. This conditioned matrix was used to examine cathepsin D–mediated collagen degradation by human breast cancer cell lines. Our results indicate that secretion of Maspin and its deposition into the extracellular milieu play an important role in matrix degradation. In this capacity, Maspin could potentially regulate mammary tissue remodeling occurring under normal and pathologic conditions. In addition, these findings could have a potential effect on future therapeutic intervention strategies for breast cancer. [Cancer Res 2007;67(8):3535–9]

Introduction

Remodeling of extracellular matrix is an innate part of normal biological processes. The dynamic interaction between multiple cell types and the extracellular matrix is crucial for mammary epithelium infrastructure, cellular homeostasis, and tissue remodeling. Altered degradation and deposition of extracellular matrix presumably by cancer cells and the surrounding stroma are hallmarks of tumor progression. The secreted proteolytic enzymes (from both the cancer cells and stromal environment) are believed to degrade the extracellular matrix, thus facilitating cancer cell invasion and metastasis (1). The lysosomal aspartyl proteinase cathepsin D is among the enzymes implicated in this process (2, 3). Under normal conditions, <20% of cathepsin D is secreted as the pro-form (pro-cathepsin D; ref. 4); however, in pathologic conditions, such as cancer, cathepsin D is aberrantly secreted and excessively produced (3, 5), and its plasma concentration is elevated in patients with metastatic breast cancer (6, 7). The secreted proteases are usually inactive pro-enzymes often associated with endogenous inhibitors, but the lower extracellular pH maintained by cancer cells (8, 9) could induce generation of the active enzyme.

Maspin is a multifaceted protein with a critical role in mammary (and prostate) tissue homeostasis (10, 11). Maspin is predominantly cytoplasmic but also localizes to other cellular compartments and is secreted (12, 13). Although the involvement of intercellular and membrane associated Maspin in myriad biological functions has been extensively studied (13), to date, the function and fate of secreted Maspin have remained enigmatic. Studies from our laboratory indicated extracellular association of Maspin with pro-cathepsin D;[3] thus, we hypothesized that the interaction of the secreted forms of Maspin and pro-cathepsin D could be a highly regulated and finely tuned process with a significant role in the extracellular matrix remodeling central to the mammary tissue homeostasis. Secreted Maspin could bind to extracellular matrix components, such as collagen, and hence alter the susceptibility of matrix to proteolytic degradation. In the absence of Maspin (as is the case in breast cancer), this balance is shifted and could lead to excessive matrix degradation and ultimately metastasis. By using normal mammary epithelial cells, we report that Maspin secreted by these cells is incorporated into the extracellular matrix. This Maspin-conditioned matrix can regulate cathepsin D–mediated matrix degradation by breast cancer cells and alter the metastatic phenotype.

Materials and Methods

Cell culture. Two normal human mammary epithelial cells (N1330 HMEC and HMEpC) were from BioWittaker, Inc. (Walkersfield, MD) and Cell Applications, Inc. (San Diego, CA) and were maintained in RPMI containing 10% FCS. Cultures were determined to be *Mycoplasma*-free using the GeneProbe rapid detection system.

Analysis of N1330 HMEC conditioned media and deposited matrix for the presence of Maspin. Conditioned media were collected from ~70% confluent N1330 HMEC (and/or HMEpC) after 48 h in culture, centrifuged to remove cell debris, and used for Maspin detection described below. For deposited matrix studies, ~70% confluent HMEC were kept in culture for 2 weeks without passaging to allow matrix accumulation. The cells were then removed by treating the monolayer with 20 mmol/L NH_4OH

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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solution followed by several washes with PBS. Complete removal of the cells was confirmed by microscopic observation, and the remaining matrix was scraped and boiled in electrophoresis sample buffer for 5 min. Conditioned media (20–30 μL) and solubilized deposited matrix were subjected to SDS-PAGE under reducing conditions and analyzed by Western blot.

HMEC-conditioned collagen matrices. A solution of 2.5 mg/mL of rat tail collagen I (BD Biosciences, Bedford, MA) and 1 mg/mL human collagen IV (Sigma, St. Louis, MO) in acetic acid was used to prepare three-dimensional collagen matrices (1–2 mm thickness) in wells of 96-well culture dish. This was achieved by neutralizing collagen solution with 20 mmol/L HEPES buffer (pH 7.4) and 0.1 mol/L NaOH solution followed by incubation at 37°C. HMEC were plated on these collagen matrices at 10^4 per well. After 4 to 5 days in culture, the cells were removed by treatment with 20 mmol/L NH_4OH solution followed by several washes with PBS. These collagen matrices (referred to as HMEC-conditioned collagen matrix) were either tested for Maspin deposition or were employed to examine the response of breast cancer cells (MCF-7 and MDA-MB-231) to signals deposited by HMEC. Breast cancer cells were plated at 10^4 per HMEC-conditioned collagen matrix in 96-well culture dishes in RPMI containing Mito$^+$ serum supplement. After 5 to 7 days, cancer cells were also removed from the collagen matrices by treatment with 20 mmol/L NH_4OH solution. The discs were washed with PBS, boiled in electrophoresis sample buffer, centrifuged at 12,000 rpm, and subjected to SDS-PAGE and Western blot analysis using antibodies to Maspin, cathepsin D, β-actin, laminin, and fibronectin. Collagen matrices without cells and with HMEC, MDA-MB-231, or MCF-7 cells served as controls.

In some experiments, recombinant Maspin (r-Maspin) or its mutant forms [Lys^{346} mutated to histidine (K/H-Maspin), tyrosine residue (Tyr^{357}) mutated to phenylalanine (Y/F-Maspin), and Arg^{341} to alanine (R/A Maspin)] were incorporated into collagen matrix at concentrations ranging from 100 to 500 ng per well. MDA-MB-231 breast cancer cells were then plated on these Maspin-incorporated matrices at 50×10^3 per well and analyzed as stated above.

Quantitative assay for collagen degradation in matrix. For quantification of matrix degradation in the presence or absence of Maspin, the matrix was supplemented with fluorescein conjugated type I collagen (DQ collagen, Molecular Probe, Eugene, OR). Degradation of collagen by proteases releases the quenched fragment that is then quantitated by FluoStar Optima ELISA reader (BMG Labteck GmbH, Durham, NC) using 485-μm emission and 530-μm excitation wavelengths. The involvement of cathepsin D in the degradation process was confirmed using specific aspartyl protease inhibitor pepstatin. Breast cancer cells were first incubated in the media containing pepstatin (200 ng/mL) for 30 min and plated on the collagen matrices in the presence of pepstatin.

Immunohistochemical analysis. Collagen matrices were prepared in Lab-Tek Chamber Slide System (Nalge Nunc International, Naperville, IL) with or without the addition of r-Maspin. MDA-MB-231 breast cancer cells or HMEC were plated on these three-dimensional matrices for 3 to 5 days. The matrix after the removal of MDA-MB-231 cells was fixed in ice-cold methanol, blocked, and treated with antibodies to cathepsin D.

Semiquantitative PCR. The effect of HMEC-conditioned matrix (or Maspin-incorporated matrix) on *cathepsin D* gene expression of breast cancer cells was examined by culturing MDA-MB-231 (and/or MCF-7) cells on pre-conditioned matrix for 0 to 4 days. Total RNA was reverse transcribed using the Advantage PCR kit (Clontech, Palo Alto, CA). Semiquantitative PCR was done using cathepsin D–specific primers (Integrated DNA Technologies, Coralville, IA). The products were separated on a 1% agarose gel with glyceraldehyde-3-phosphate dehydrogenase as loading control.

Results

Maspin secretion by normal mammary epithelial cells. Our studies indicate that HMEC maintained over 1 to 2 weeks without passaging deposit a significant amount of matrix (deposited matrix) comprised of laminin, fibronectin, and actin (Supplementary Fig. S1). Based on our previous studies,[3] these cells also secrete Maspin. Therefore, we asked whether any of the secreted Maspin becomes incorporated into this deposited matrix. As shown in Fig. 1*A*, Maspin is identified in N1330 HMEC (and HMEpC)

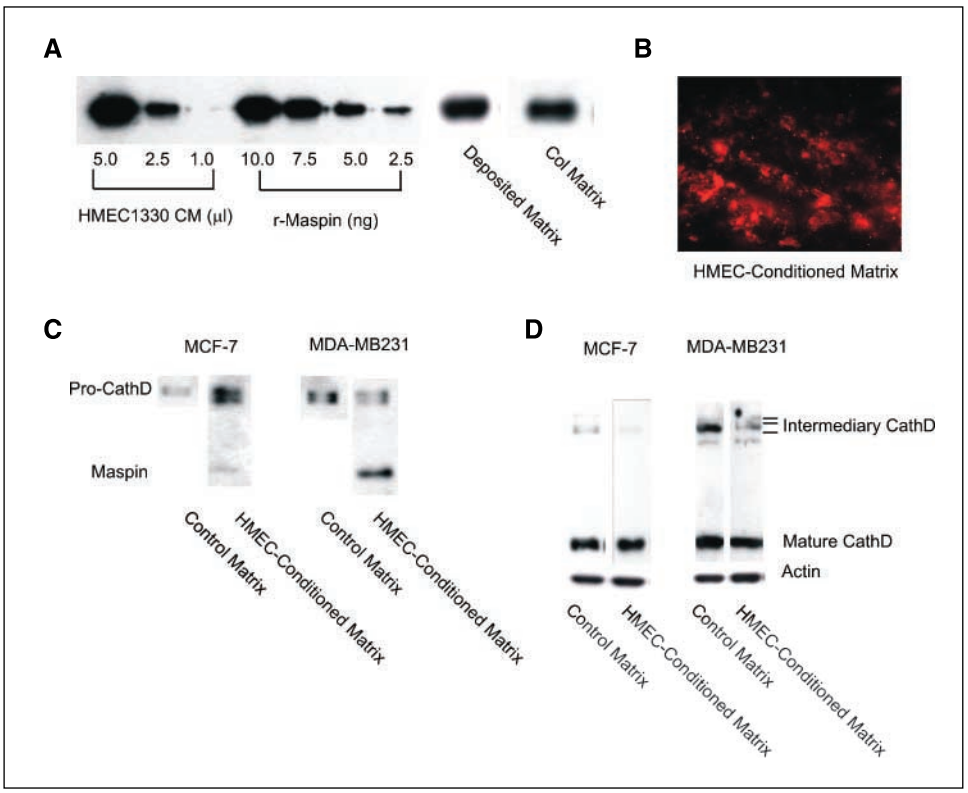

Figure 1. *A,* Western blot analysis to detect Maspin in the conditioned medium (*CM*) and the matrix deposited (*Deposited matrix*) by normal mammary epithelial cells (HMEC 1330) and its incorporation into a three-dimensional collagen (*Col*) matrix (HMEC-conditioned matrix). r-Maspin at concentrations indicated was used to examine the level of Maspin secreted into the conditioned medium and/or deposited in the matrix. *B,* the presence of Maspin in the HMEC-conditioned matrix was further confirmed by immunohistochemical analysis of the matrix following the complete removal of the cells. Breast cancer cell lines MCF-7 and MDA-MB-231 were plated on collagen I matrix in 96-well culture dishes (75×10^3 per well) for 4 d. *C,* the conditioned medium was collected and concentrated 10× (MCF-7) or 2× (MDA-MB-231) and subjected to Western blot analysis to detect secreted pro-cathepsin D (*pro-CathD*) and Maspin. *D,* the cells were then removed by treatment with 20 mmol/L NH_4OH; the remaining collagen matrices were washed with PBS and boiled in SDS-PAGE sample buffer and subjected to Western blot analysis for the detection of cathepsin D. The cathepsin D detected in the conditioned medium is the pro-form (~52–54 kDa), whereas that in the matrix is the intermediary and mature active forms.

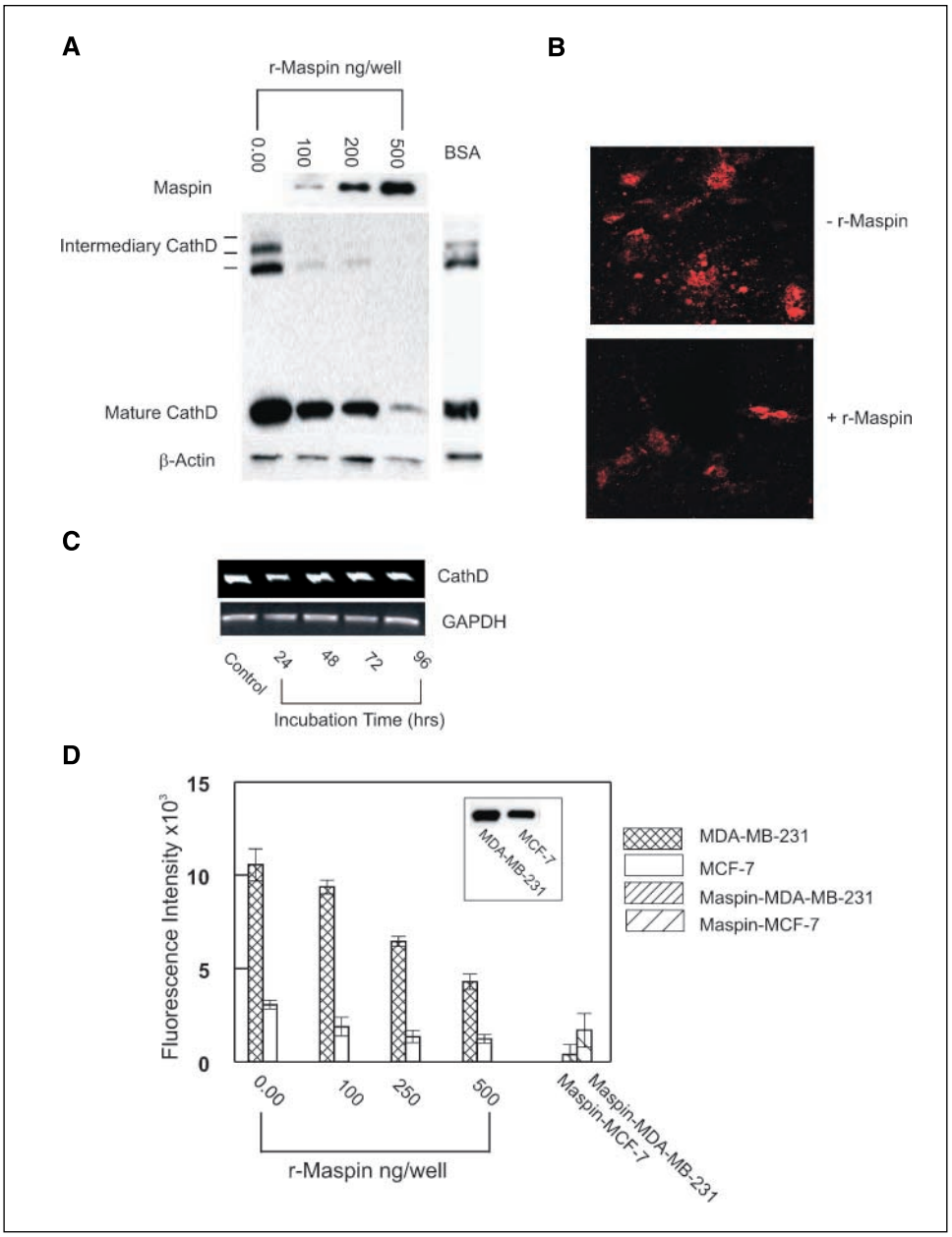

Figure 2. *A,* r-Maspin was incorporated into collagen solution before the gelation process at concentrations ranging from 100 to 500 ng per well in 96-well culture dishes. MDA-MB-231 cells were plated on these gels as described in Fig. 1 legend. Analysis of the matrix by Western blot for the presence of Maspin and cathepsin D (and actin as loading control) is depicted in Fig. 1*A*. For comparison purposes, bovine serum albumin (*BSA*)–incorporated (500 ng per well) collagen matrix is also included and indicates minimal changes in cathepsin D levels. *B,* for immunohistochemical analysis, collagen matrices were cast in an eight-well culture dish with or without r-Maspin (500 ng per well). MDA-MB-231 cells were plated on these matrices at 10^5 per well for a period of 4 d. The cells were then removed by NH_4OH treatment (as described in the Materials and Methods), and the remaining matrices were fixed and treated with antibodies to cathepsin D. *C,* culturing MDA-MB-231 breast cancer cells on Maspin-incorporated collagen matrix is associated with a reduction in cathepsin D mRNA within 24 h, which returns to baseline by 48 h. *D,* for quantitative estimation of collagen matrix degradation, fluorescein-conjugated collagen I (DQ collagen) was incorporated into the matrix, and the fluorescent fragments released from the proteolytic cleavage of collagen I were quantified by an ELISA reader. For comparison, collagen matrix degradation resulting from Maspin-transfected MCF-7 or MDA-MB-231 are included. *Inset,* Western blot analysis of post-nuclear fraction of Maspin-transfected MCF-7 and MDA-MB-231 cells to show Maspin expression.

conditioned media and is incorporated into the deposited matrix. Both normal mammary epithelial cell lines gave comparable results; thus, for simplicity sake in the following text, they are referred to collectively as HMEC. In addition, when HMEC were plated on collagen matrices, the deposition of Maspin in the HMEC-conditioned collagen matrix was confirmed by Western blot and immunohistochemical approaches (Fig. 1*A* and *B*).

Maspin inhibits cathepsin D–mediated collagen matrix degradation. To examine the significance of Maspin incorporation into collagen matrix and its possible role in maintaining matrix integrity, MDA-MB-231 (or MCF-7) cells were plated either on collagen matrices or HMEC-conditioned collagen matrices. After 4 to 5 days in culture, the conditioned medium was collected; the cells were removed; and the remaining denuded collagen matrices were subjected to SDS-PAGE under reducing conditions. Western blot analysis of the conditioned media and the collagen matrices indicated the presence of pro-cathepsin D (52–54 kDa) in the conditioned media of all the wells tested (Fig. 1*C*). Interestingly, collagen matrix–associated cathepsin D was mostly the intermediary (intermediary cathepsin D; 48 and 43 kDa) and mature active (mature cathepsin D; 34 kDa) forms (Fig. 1*D*). Although the pro-cathepsin D levels in the conditioned media were minimally divergent, cathepsin D levels (specifically intermediary cathepsin D) in the HMEC-conditioned matrices were lower than that of control. As anticipated, Maspin was present in the HMEC-conditioned matrices, and it was also detected in the conditioned media of the wells, which contained these matrices, presumably released by the proteolytic degradation of the matrix (Fig. 1*C*).

To confirm that changes in cathepsin D levels of MDA-MB-231 cells were solely due to Maspin and not other secreted products of HMEC, MDA-MB-231 cells were cultured on collagen matrix containing r-Maspin at concentrations ranging from 100 to

500 ng. A concentration-dependent decrease in collagen matrix–associated cathepsin D was observed (Fig. 2*A*); substitution of Maspin with bovine serum albumin in collagen matrix had minimal effect on cathepsin D–associated collagen matrix (Fig. 2*A*). These observations were further supported by our immunohistochemical analysis of the control and Maspin-incorporated collagen matrices following exposure to MDA-MB-231 cells, which revealed reduced deposition of cathepsin D in r-Maspin–incorporated matrices compared with control (Fig. 2*B*). In addition, reverse transcription-PCR analysis of MDA-MB-231 mRNA from cells cultured on control and Maspin-incorporated matrices indicated that the latter exerted a modest suppressive effect on *cathepsin D* gene expression within 24 h, which returned to normal by 48 h (Fig. 2*C*).

It is noteworthy that compared with the HMEC-deposited Maspin, a much higher concentration of r-Maspin was required to exert a similar inhibitory effect. Thus, we purified Maspin from HMEC conditioned media (using polyclonal anti-Maspin) and examined its ability to inhibit matrix degradation by MDA-MB-231 cells. This approach gave similar results to those obtained with r-Maspin (Supplementary Fig. S2).

Further confirmation for the ability of Maspin to alter matrix degradation was obtained by incorporating DQ collagen into the matrix. Quantifying the released fluorescent fragments indicated that Maspin reduces the release of fluorescent fragments (and hence generally inhibits degradation of matrix) in a concentration-dependent manner (Fig. 2*D*).

Requirement for the intact reactive center loop of maspin. By using mutated r-Maspins, two with a mutation in the reactive center loop (RCL; Arg^{341} to alanine [R/A] and Lys^{346} to histidine [K/H]) and one at Tyr^{357} [Y/F], we observed that these mutations constrain the ability of Maspin to inhibit cathepsin D–mediated matrix degradation (Fig. 3*B*). Interestingly, R/A-Maspin and, to some extent, K/H-Maspin did not fully bind to collagen matrix and were recovered mostly in the conditioned media collected following the completion of the experiments (Fig. 3*A*).

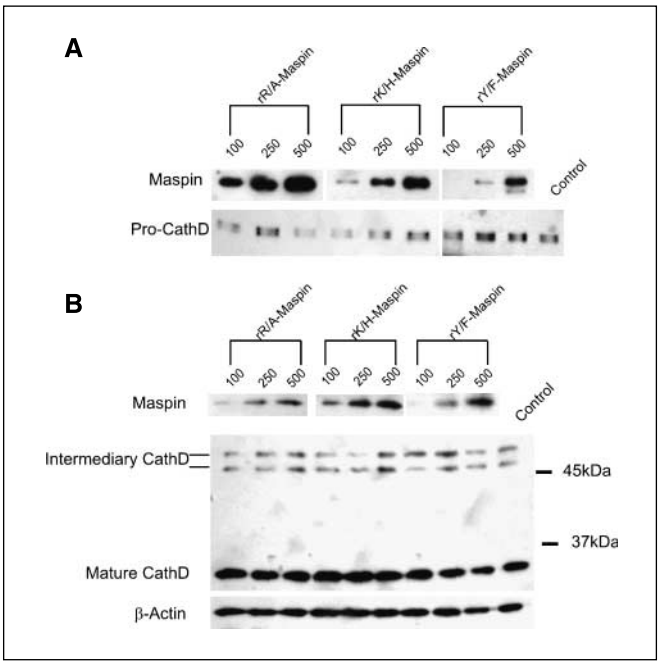

Figure 3. Three different recombinant mutated Maspin(s) were incorporated into collagen matrix as described in Fig. 2 legend. MDA-MB-231 cells were plated on these matrices in 96-well culture dishes (75×10^3 per well) for 4 d. *A,* the conditioned medium was collected, concentrated 2×, and subjected to Western blot analysis to detect secreted pro-cathepsin D and r-mutated Maspin. *B,* the cells were removed by treatment with 20 mmol/L NH_4OH, and the denuded matrices were washed with PBS and boiled in SDS-PAGE sample buffer and subjected to Western blot analysis for the detection of matrix-associated cathepsin D and r-mutated Maspin. Actin was used as loading control. *rR/A-Maspin,* recombinant Maspin mutated at Arg^{341} to alanine; *rK/H-Maspin,* recombinant Maspin mutated at Lys^{346} to histidine; *rY/F-Maspin,* recombinant Maspin mutated at Tyr^{357} to phenylalanine (generated in our laboratory). The position of 45- and 37-kDa molecular weight markers are indicated.

Discussion

Although the secretion of Maspin by mammary epithelial cells (detected by Western blot analysis) has been known for some time (12, 13), its function in the extracellular milieu has remained mostly unexplored. As a prelude to understanding this aspect of Maspin, we examined the HMEC conditioned media for the presence of this protein. Our studies indicated that the secreted Maspin has a molecular mass similar to the cell associated one (42 kDa) and is incorporated into the matrix deposited by these cells. In addition, upon culturing HMEC on collagen matrix, the secreted Maspin is incorporated into three-dimensional collagen matrix. Apart from Maspin, other cellular proteins, such as laminin, fibronectin, actin, and collagen IV, were also detected in both deposited matrix and the HMEC-conditioned matrix, thus suggestive of an important role for epithelial cells in assembling basement membrane matrix. The ability of Maspin to bind collagen is further confirmation of the studies by Blacque and Worrall, identifying collagen I and III as the binding partners of Maspin (14), and could indicate a biological role for Maspin in matrix remodeling. In our study, this was examined by culturing the breast cancer cell line MDA-MB-231 on the HMEC-conditioned matrix and examining its ability to degrade matrix. The results indicated that an HMEC-conditioned collagen matrix is less susceptible to cathepsin D–mediated degradation by breast cancer cells. By using r-Maspin and/or Maspin purified from the conditioned media of HMEC, we established a role for Maspin in this process. Interestingly, compared with the HMEC-deposited Maspin, a much higher concentration of r-Maspin, or purified Maspin protein, was required to give a comparable inhibition of cathepsin D–mediated matrix degradation. It is plausible to speculate that the "positional" deposition of Maspin in the matrix, or its specific association with other proteins secreted by HMEC, could be a determining factor.

Our studies indicate that the intact RCL of Maspin plays a role in reducing cathepsin D–mediated matrix degradation, as mutation(s) in the RCL diminish(es) the inhibitory effect. This finding further supports the importance of intact Maspin RCL in adhesion, migration, and motility (11, 13, 15). Specifically, Maspin/ovalbumin chimeric proteins have established the necessity of an intact Maspin RCL for adhesion to matrix of corneal stromal cells and MDA-MB-231 breast cancer cell lines (16). In the same context, the R/A mut-Maspin even fails to fully adhere to our collagen matrix and is recovered mostly in the conditioned media. Thus, our findings of the inhibitory effect of Maspin on cathepsin D–mediated matrix degradation might also partially reflect the necessity of Maspin interaction with, and attachment to, matrix. It is possible that the incorporated Maspin binds to both collagen and the surface of MDA-MB-231 cells (16) and alters their ability to degrade matrix. The observed failure of Maspin with mutations in the RCL to inhibit cathepsin D–mediated matrix degradation might result from its

inability to bind to both collagen and MDA-MB-231 cancer cells. Our study reveals that the presence of Maspin in the matrix clearly deters cathepsin D incorporation; however, it does not indicate a direct effect of Maspin on cathepsin D. Whether Maspin and cathepsin D bind to the same site in collagen remain to be determined.

Although cathepsin D secreted by MDA-MB-231 cells and detected in the conditioned media was pro-cathepsin D, its collagen matrix–associated counterpart was comprised of intermediary and mature cathepsin D forms. It is possible that the pro-cathepsin D released by cancer cells and bound to matrix undergoes autoactivation juxtaposed to the cells and in response to the more acidic pH maintained by cancer cells (8, 9, 17). Alternatively, as has been reported for cysteine proteases, such as cathepsin B and L, the more acidic pH of the tumor microenvironment could induce endosomal (lysosomal) mobilization and release of active cathepsin D into the matrix (18, 19).

In conclusion, our studies indicate for the first time that secreted Maspin is incorporated into the matrix deposited by HMEC and an artificial three-dimensional collagen matrix and thus plays a critical role in matrix remodeling. In addition, the presence of Maspin in collagen matrix deters matrix incorporation of cathepsin D, thereby reducing matrix degradation.

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Correction: Elucidating the Function of Secreted Maspin

In the article on elucidating the function of secreted Maspin in the April 15, 2007 issue of *Cancer Research* (1), the paragraph entitled "Cell culture" on page 3535 should have read as follows:

Cell culture. Two normal human mammary epithelial cells (N1330 HMEC and HMEpC) were from BioWittaker, Inc. (Walkersfield, MD) and Cell Applications, Inc. (San Diego, CA) and were maintained in defined mammary epithelial cell medium provided by the respective companies. All other cell lines used were maintained in RPMI containing 10% FCS. Cultures were determined to be *Mycoplasma*-free using the GeneProbe rapid detection system.

1. Khalkhali-Ellis Z, Hendrix MJC. Elucidating the function of secreted Maspin: inhibiting cathepsin D-mediated matrix degradation. Cancer Res 2007;67:3535–9.

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