Expression of Membrane Type 1 Matrix Metalloproteinase Is Associated with Cervical Carcinoma Progression and Invasion

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Abstract
Membrane type 1 matrix metalloproteinase (MT1-MMP) is frequently expressed by cancer cells and is believed to play an important role in cancer cell invasion and metastasis. However, little is known about the role of MT1-MMP in mediating invasiveness of cervical cancer cells. In this study, we examined MT1-MMP expression in 38 primary human cervical tissue specimens, including normal cervix, low-grade squamous intraepithelial lesions (LSIL), high-grade SILs (HSIL), and invasive carcinomas. We also evaluated MT1-MMP, MMP-2, and tissue inhibitor of metalloproteinase-2 expression in several cervical cancer-derived cell lines, human papillomavirus (HPV)-immortalized keratinocytes, and keratinocytes derived from a LSIL. Using in situ hybridization techniques to study the cervical tissue specimens, we found that MT1-MMP expression increases with cervical tumor progression (Spearman correlation coefficient = 0.66; P < 0.0001, exact test). Specifically, MT1-MMP expression is very low or absent in normal cervix and LSILs, is readily detectable in HSILs, and is very strongly expressed in nearly all invasive carcinomas. Most but not all cervical cancer-derived cell lines also expressed significant levels of MT1-MMP and MMP-2. Constitutive expression of exogenous MT1-MMP in cervical carcinoma-derived cells and HPV-immortalized keratinocytes with low endogenous levels of MT1-MMP induced invasiveness in collagen I, but this effect was not observed in LSIL-derived keratinocytes. Our results show that MT1-MMP is a key enzyme mediating cervical cancer progression. However, MT1-MMP alone is not always sufficient for inducing keratinocyte invasiveness at least in the collagen I invasion assay used in this study. Further studies of gene expression in preinvasive and invasive cervical cancers should assist with identification of additional critical factors mediating cervical cancer progression.

Introduction
Cervical cancer is the third most common cancer in women, with ~371,200 new cases diagnosed each year worldwide (1). Human papillomavirus (HPV) DNA sequences are present in the vast majority of cervical carcinomas, and multiple complementary lines of evidence support a causal role for infection with certain HPV types in cervical cancer pathogenesis (2). Overall, the ratio of mortality to incidence is 51% and cervical cancer thus remains a significant public health concern (1). Cervical cancer development has been linked to intraepithelial precursor lesions called squamous intraepithelial lesions (SIL). Estimates of the prevalence of SILs range from 0.5% to 6.5% of the U.S. female population, including >50,000 new cases of carcinoma in situ each year. However, it is estimated that only 12% to 22% of high-grade SILs (HSIL) will progress to invasive carcinoma if left untreated (3–5). Morphologic examination alone does not allow distinction of those HSILs likely to progress from those that will regress or simply persist (3). Clearly, a better understanding of the molecular mechanisms by which preinvasive lesions acquire the ability to invade the cervical stroma and ultimately metastasize would have a significant clinical impact.

In order for progression from preinvasive to invasive carcinoma to occur, neoplastic epithelial cells must acquire the ability to penetrate the basement membrane and degrade the underlying extracellular matrix (ECM), which is composed of several components, including collagen, elastin, and fibronectin (6). Matrix metalloproteinases (MMP) are believed to play an important role in the process of tumor invasion via their ability to degrade many of these ECM components (7–9). Elevated expression of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) has been reported in several types of human cancer, including cervical carcinoma (10–15). MMP-2 and MMP-9, like most other MMPs, are secreted as inactive proenzymes that undergo activation following cleavage of an NH2-terminal prodomain (reviewed in ref. 16). The membrane type MMP known as MT1-MMP (MMP-14) can degrade several components of the ECM, including fibronectin, vitronectin, fibrin, laminin 1 and 5, and collagen I, II, and III (17–19). MT1-MMP is also a specific activator of pro-MMP-2 at the cell surface. MT1-MMP participates in the activation of pro-MMP-2 through formation of a trimolecular complex with tissue inhibitor of metalloproteinase (TIMP)-2 and pro-MMP-2. Once the ternary complex is formed, another MT1-MMP molecule in the homooligomeric complex cleaves the NH2-terminal prodomain of pro-MMP-2, thus generating an intermediate that matures into the fully active MMP-2 enzyme through an autoprotoeolytic mechanism (16, 19–23). Consequently, MT1-MMP is considered a key enzyme that contributes to tumor cell invasion and metastasis through direct ECM degradation and/or activation of downstream MMPs, such as pro-MMP-2 (18, 24). In turn, MT1-MMP activity can be either inhibited following interaction with tissue inhibitors of metalloproteinases (TIMP) or destroyed as a consequence of autoprotoeolytic or lysosomal degradation (21, 24–29).

Elevated expression of MT1-MMP has been observed in cancers of the lung, colon, liver, breast, brain, head and neck, ovary, and...
uterine cervix (10, 30–38). To better characterize the role of MT1-MMP in cervical cancer pathogenesis, we evaluated expression of MT1-MMP in 58 primary cervical tissue specimens spanning the spectrum from normal cervix to invasive carcinoma. We also characterized expression of MMP-2, MT1-MMP, and TIMP-2 in a panel of cell lines derived from cervical carcinomas, SILs, and HPV-immortalized keratinocytes and tested the ability of these cells to invade collagen I. Finally, we directly assessed the role of MT1-MMP activity in mediating invasive behavior of neoplastic squamous epithelial cells.

Materials and Methods

Tumor samples. A total of 58 paraffin-embedded cervical tissue specimens were analyzed. Nine were obtained from the surgical pathology archives of the Johns Hopkins Hospital, 2 from the University of Michigan Hospital, and 47 from a previous study conducted in Spain and Colombia (39–41). H&E-stained sections from each specimen were histologically verified by a gynecologic pathologist (K.R.C.) as squamous cell carcinoma (n = 42), HSIL (n = 5), low-grade SIL (LSIL; n = 4), or normal cervix (n = 7). Analysis of tissues from human subjects was approved by the University of Michigan Institutional Review Board (IRB-MED 2002-0430).

Cell lines and cell culture. Seven cervical carcinoma–derived cell lines (C-33A, C-4II, ME-180, CaSkii, MS571, HT-3, and HeLa) and one fibrosarcoma-derived cell line (HT-1080) were obtained from the American Type Culture Collection (Manassas, VA). The HPV16-immortalized keratinocyte cell line 8217 was a gift from P. Hawley Nelson (National Cancer Institute, Bethesda, MD). The HPV18-immortalized keratinocyte cell line 1811 and its NMu-transformed counterpart (NMU-T1) were a gift from J.K. McDougall (Fred Hutchinson Cancer Research Center, Seattle, WA; refs. 42, 43). Cervical intraepithelial neoplasia (CIN) 612 [derived from grade 1 (CIN1; i.e., LSIL)] and the HPV18-immortalized cervical keratinocyte cell line 610 were a gift from K. De Geest (Rush Medical College, Chicago, IL; ref. 44). ME-180 and HT-3 cells were cultured in McCoy’s 5A medium (Invitrogen Corp., Grand island, NY) with 10% fetal bovine serum (FBS; Invitrogen). CaSkii cells were cultured in RPMI 1640/10% FBS. Keratinocyte-derived cells (NMU-T1, 1811, 8217, 610, and CIN612) were cultured in keratinocyte growth medium (Cambrex, Walkersville, MD). All other cell lines were maintained in DMEM with 10% FBS.

In situ hybridization detection of membrane type 1 matrix metalloproteinase expression. A cDNA fragment spanning MT1-MMP nucleotides 2,483 to 2,884 was subcloned into the pPST18 and pPST19 vectors (Roche Diagnostics GmbH, Indianapolis, IN). Tissue sections were incubated with hybridization solution with no probe. After rinsing with PBS, sections were treated with 0.1 mol/L HCl at 37°C for 10 minutes at room temperature. Permeabilization with a TE buffer for 10 minutes. Sections were incubated with prehybridization buffer for 2 hours at 45°C. Following an additional 24-hour incubation at 37°C, the slides were placed in blocking solution (Roche Diagnostics Ltd., Welwyn, United Kingdom) containing 0.5% bovine serum albumin for 30 minutes at room temperature, and then incubated with anti-digoxigenin Fab fragments at 1:500 dilution in blocking solution overnight at 4°C in a humid chamber. Hybridization signals were detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate after development in the dark for 4 to 8 hours. The color reaction was terminated by incubating the slides in TE buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0)]. Finally, sections were counterstained for 1 to 2 minutes with 0.1% nuclear fast red. MT1-MMP expression was scored as strong (+), moderate (+), or weak/absent (−) based on signal intensity in the epithelial cells.

Expression vector construction and transfection. Full-length MT1-MMP cDNA (spanning nucleotides 226-1,983, Genbank accession no. NM-004995) was generated by reverse-transcription PCR using total RNA from HT-1080 human fibrosarcoma cells. The PCR primers were designed to include a Flag epitope tag at the MT1-MMP COOH terminus. PCR products were subcloned into the retroviral vector pPGS-cytomelicoravirus-CITE-neo (45) and the cdna sequence of individual clones was verified by automated DNA sequencing. Selected cell lines (CIN612, 1811, HeLa, C-33A, and CaSkii) were transduced with retroviral supernatant from amphotrophic Phoenix cells transfected with vector alone or vector with MT1-MMP. Stable polyclonal lines were generated by selection in G418 at a concentration of 0.4 to 1 mg/mL. After 1 week, the G418 concentration was reduced to 0.2 to 0.4 mg/mL, and expression of Flag-tagged MT1-MMP protein was confirmed by Western blot analysis.

Western blot and gelatin zymography. Parental and stably transduced cells were lysed in cold Triton X-114 lysis buffer containing proteinase inhibitors (complete proteinase inhibitors; Roche Applied Science, Indianapolis, IN; ref. 46). Whole-cell lysates were analyzed by Western blotting with anti-MT1-MMP monoclonal antibody (Calbiochem, San Diego, CA) at 1:500 or anti-Flag M2 antibody (Sigma, St. Louis, MO) at 1:5,000. Expression of β-actin was used as a loading control and detected with anti-actin polyclonal antibody (Sigma). For evaluation of secreted MMP-2 and TIMP-2, conditioned medium was collected from cells cultured for 24 hours in serum-free conditions. Medium was concentrated 20-fold and then stored at −70°C until use. Concentrated conditioned medium was analyzed by Western blot with anti-MMP-2 antibody (Calbiochem) at 1:100 and anti-TIMP-2 (Calbiochem) at 1:400. Gelatinolytic activity was examined by gelatin zymography. Concentrated conditioned medium was resolved by electrophoresis in polyacrylamide gels impregnated with 2 mg/mL gelatin. SDS was removed by washing the gel with 2.5% Triton X-100 buffer for 1 hour. The gels were then incubated at 37°C in 0.1 mol/L Tris-HCl, 10 mmol/L CaCl2, 5 µmol/L ZnCl2, 0.0015% Brij-35 for 18 hours and stained with Coomassie brilliant blue R250 (47). Gelatinolytic activity was detected as clear bands against a blue background.

Invasion assays. Invasion assays were done as described previously by Hotary et al. (46). In brief, rat tail collagen I (Sigma) was dissolved in 0.2% acetic acid to a final concentration 2.3 mg/mL. To induce gelling, collagen was mixed with 10× DMEM and 0.34 N NaOH in an 8:1:1 ratio at 4°C, and 1 mL of this mixture was added to the upper well of 24 mm Transwell dishes (3 µm pore size; Corning Costar Corp., Corning, NY). After gelling was completed (45 minutes at 37°C), complete medium (2 mL) was added to the lower well and 1 × 105 cells in complete medium were added to upper well. Following an additional 24-hour incubation at 37°C, epidermal growth factor (EGF) was added to the lower compartment of selected Transwell chambers at a final concentration of 10 ng/mL (48, 49). The cells were grown on top of collagen gels for 12 days and medium was exchanged every 2 to 3 days. Gels were fixed in 4% paraformaldehyde in PBS, removed from the Transwells, embedded in paraffin, and stained with H&E. Invasion was determined using a light microscope at ×400 magnification.

Results

Membrane type 1 matrix metalloproteinase expression in cervical tissue specimens. In an effort to determine whether increased MT1-MMP expression is associated with cervical cancer invasion, we used in situ hybridization to detect MT1-MMP transcripts in normal cervical tissue and several samples of LSIL, HSILs, and invasive squamous cell carcinomas. As indicated in Table 1, nearly all invasive carcinomas showed strong (n = 17) or...
moderate \((n = 24)\) MT1-MMP expression (Fig. 1A-C). Expression of MT1-MMP was weak/absent in only 1 of the 42 carcinoma specimens. HSILs generally expressed somewhat lower levels of MT1-MMP than the carcinomas, with four cases showing moderate expression and one case showing strong expression (Fig. 1D-F). Notably, MT1-MMP expression was weak or absent in all LSILs and normal cervical tissues (Fig. 1G-I). Considering the relatively small sample size and zero-containing cells in Table 1, we conducted the exact test using Spearman correlation coefficient to test the monotone trend association between MT1-MMP expression and cervical tumor progression. The association between increased MT1-MMP expression and cervical tumor progression is indeed statistically significant (Spearman correlation coefficient = 0.66; \(P < 0.0001\), exact test, two-sided). These findings indicate that MT1-MMP expression increases during progression from preinvasive to invasive cervical cancer and suggest that MT1-MMP may be a necessary factor for conferring the invasive phenotype in neoplastic cervical epithelial cells.

Membrane type 1 matrix metalloproteinase, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression in cervical cancer cell lines. We used immunoblots and/or gelatin zymography to characterize expression of MT1-MMP, MMP-2, and TIMP-2 in eight human cervical cancer-derived cell lines (SiHa, ME-180, HT-3, C-33A, C-4II, CaSki, MS751, and HeLa) and four HPV-immortalized keratinocyte cell lines (NMU-T1, 1811, 8217, and 610) and CIN1-derived cell line CIN612. HT-1080 cell lysates were used as positive control. As shown in Fig. 2A, MT1-MMP protein was detectable in most of the cell lines, with highest expression in NMU-transformed, HPV18-immortalized keratinocytes (NMU-T1). Expression was undetectable in C-33A and weak in C-4II, HeLa, and CIN612 cells. Gelatin zymography was used to detect MMP-2 enzymatic activity in the same cell lines (Fig. 2B). In all cell lines, except for NMU-T1, pro-MMP-2 (72 kDa) was identified, although only the HT-1080-positive control cells showed processed MMP-2 (62 kDa) under these assay conditions. TIMP-2 was also variably expressed, with highest expression in

<table>
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<tr>
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Table 1. MT1-MMP mRNA expression in normal cervix, LSILs, HSILs, and invasive cervical carcinomas

Figure 1. **In situ** hybridization showing MT1-MMP expression in cervical carcinomas and HSILs but not in normal cervical mucosa. Representative H&E-stained sections from an invasive cervical carcinoma (A), HSIL (D), and normal cervix (G). Tissue sections from the same specimens were hybridized with digoxigenin-labeled MT1-MMP antisense (B, E, and H) and sense (C, F, and I) riboprobes. Original magnification, \(\times 400\).
SiHa, HeLa, and NMU-T1 and moderate expression in the C-33A, C-4II, and CaSki carcinoma cell lines, the HPV-immortalized but nontransformed keratinocyte cell lines, and CIN-derived keratinocytes (Fig. 2C). TIMP-2 expression was undetectable in ME-180, HT-3, and MS751 (Fig. 2C).

Membrane type 1 matrix metalloproteinase and invasion. 
MT1-MMP is thought to mediate tumor cell invasion by degrading the pericellular ECM either through direct ECM cleavage or indirectly through activation of accessory MMPs. To assess the role of MT1-MMP in conferring invasive properties to cells derived from...
frankly malignant cervical epithelial neoplasms, we first determined whether expression of endogenous MT1-MMP correlated with ability to invade collagen I. Cells from each available cervical carcinoma–derived line were grown on top of collagen I gels for 12 days in the absence or presence of EGF. Previous studies have shown that both normal and neoplastic cells can mobilize proteolytic enzymes, such as MT1-MMP and MMP-9, in response to EGF (50–52). Only SiHa cells invaded the collagen I matrix in the absence of EGF stimulation (Fig. 3A and B). With EGF stimulation, CaSki and ME-180 cells also displayed the collagen-invasive phenotype (Fig. 3C and D). Notably, all three carcinoma cell lines with markedly reduced or absent endogenous MT1-MMP expression (C-33A, HeLa, and C-4II) as well as HT-3 and MS751 failed to invade collagen I whether EGF was present or not (Fig. 3E and F). These findings suggest that MT1-MMP expression is likely necessary although not sufficient to confer invasiveness to cervical carcinoma cells in this assay system.

To determine whether ectopic expression of MT1-MMP can induce or enhance invasiveness of cervical cancer cells in collagen I, we transduced CaSki, HeLa, and C-33A cells with replication-deficient retroviruses containing a full-length MT1-MMP cDNA and selected for polyclonal cell lines that had integrated the vector. Control (empty vector) cell lines were generated in parallel. Expression of exogenous MT1-MMP was confirmed by immunoblot analysis using both anti-Flag (Fig. 4) and anti-MT1-MMP antibodies (data not shown). MT1-MMP-overexpressing and control cells were grown in the presence or absence of EGF stimulation on top of collagen I gels. Consistent with previous results, CaSki cells transduced with vector alone (CaSki.neo) showed an invasive phenotype only when stimulated with EGF (Fig. 5A and B). However, invasion was observed in a polyclonal CaSki cell line with high levels of ectopic MT1-MMP expression (CaSki-MT1-MMP) even in the absence of EGF stimulation. Invasion of the CaSki-MT1-MMP cells was further enhanced by EGF treatment (Fig. 5C and D). As expected based on studies of parental C-33A and HeLa cells, the control C-33A.neo and HeLa.neo lines failed to show an invasive phenotype with or without EGF stimulation (Fig. 5E, F, I, and J). In contrast, both C-33A-MT1-MMP and HeLa-MT1-MMP polyclonal lines ectopically expressing exogenous MT1-MMP invaded collagen in the absence of EGF stimulation, and invasiveness was further enhanced by EGF exposure (Fig. 5G, H, K, and L).

We then wished to determine whether increased MT1-MMP expression could induce invasion of nontransformed HPV-immortalized (1811) or CIN-derivated keratinocytes (CIN612) into collagen. Cells were transduced with retroviruses containing MT1-MMP or empty vector and expression of exogenous MT1-MMP in appropriate lines was confirmed by immunoblotting (Fig. 4). HPV18-immortalized 1811 cells expressing exogenous MT1-MMP displayed the ability to invade collagen I in the presence of EGF (Fig. 6A-D). However, increased MT1-MMP expression failed to induce the invasive phenotype in CIN612 cells with or without EGF stimulation (Fig. 6E-H).
Discussion

Uncontrolled expression of MMP-2 and MMP-9 is thought to be critical for conferring invasive potential to tumor cells because these proteases can degrade a wide variety of ECM components found in both basement membranes and pericellular stroma (6). Previous studies have shown that expression of MT1-MMP is generally well correlated with MMP-2 activation in various human cancers (33, 38, 52, 53), suggesting that MT1-MMP also plays an important role in cancer cell invasion and metastasis. This presumably occurs through direct ECM cleavage by MT1-MMP and perhaps via MT1-MMP-mediated activation of pro-MMP-2, pro-MMP-13, or pro-MMP-8 (19, 24, 54). Our finding that nearly all invasive primary cervical carcinomas express high levels of MT1-MMP transcripts corroborates other studies in the published literature (13, 30, 35). Although the data regarding MT1-MMP expression in cervical cancer precursor lesions are more limited, we and others have shown that HSILs typically express lower but often detectable MT1-MMP transcripts, whereas MT1-MMP expression is absent in LSILs and normal cervical tissues (30, 35). Collectively, these findings suggest that MT1-MMP plays an important, if not requisite, role in cervical cancer progression. Recent studies have shown that MT1-MMP serves as the major cell-associated protease necessary to confer normal or neoplastic cells with invasive activity independently of plasminogen, the MMP-2/TIMP-2 axis, MMP-9, collagenase-3, collagenase-4, and stromelysin-1 (54). Further, independent of regulating invasive activity, other MMPs can play important roles in directing tumor cell behavior (55). However, MT1-MMP expression alone may be insufficient to confer an invasive phenotype to cervical epithelial cells in some settings. For example, several inhibitors of MT1-MMP activity have been identified, including TIMP-2, testican 1 and 3, RECK, and the Cupin superfamily member MTCBP-1 (29, 56–58). Moreover, the absence of detectable MT1-MMP expression in at least one cervical carcinoma–derived cell line (C-33A) and one of our primary carcinomas suggests that cervical carcinomas can occasionally arise in the absence of MT1-MMP overexpression or, alternatively, can lose expression of MT1-MMP once they are established in vitro. It is also possible that MT2-MMP (MMP-15) may function in lieu of (or in addition to) MT1-MMP in conferring invasive activity to some cancers (46). Notably, Sheu et al. recently reported a comprehensive analysis of MMPs in human cervical cancers (30). Using immunohistochemistry, they found MT1-MMP and MT2-MMP expression in 81% and 65% of invasive cervical carcinomas, respectively.

We found that cervical carcinoma cells with absent or low endogenous MT1-MMP (C-33A, C-4II, and HeLa) did not exhibit an invasive phenotype in collagen I even in the presence of EGF. Ectopic expression of MT1-MMP in each of these cell lines resulted in collagen I invasiveness that was further enhanced by EGF. Thus, in these cells, it is likely that endogenous MT1-MMP expression is insufficient to allow degradation of collagen I in vitro. We also found that two cell lines expressing significant endogenous MT1-MMP (HT-3 and MS751) failed to exhibit invasiveness in our assay, although we cannot exclude the possibility that MT1-MMP activity is suppressed in these cells by endogenous inhibitors, such as testican 3, RECK, or MTCBP-1. In addition, further studies are needed to exclude the possibility that these cells are unable to mobilize the complex cell surface and intracellular machinery required to support two-dimensional and three-dimensional motility (59).

Along these lines, it is notable that HPV-immortalized keratinocytes (e.g., 1811 cells) transduced with retroviruses allowing high-level expression of MT1-MMP displayed invasive behavior in the presence of EGF stimulation. In contrast, similarly transduced cells derived from a LSIL (CIN1) positive for HPV31b failed to invade collagen I even in the presence of EGF. These findings are consistent with the notion that MT1-MMP expression, in and of itself, may not be sufficient to induce keratinocyte invasion, perhaps because other required cofactors are absent or MT1-MMP inhibitors are expressed.

As part of their analysis of MMPs in human cervical cancers, Sheu et al. assessed MMP-2 and MMP-9 gelatinolytic activity in microdissected primary tumor specimens (30). They found progressively up-regulated expression of MMP-2 and MMP-9 in cervical cancer progression and close correlation of MMP-2 and MT1-MMP expression in primary tumor specimens. MMP-2 and MMP-9 gelatinolytic activity was significantly associated with tumor stage, nodal metastasis, and tumor recurrence. These findings suggest
that therapies targeting MMP-2 and MMP-9 activity may prove efficacious for treating cervical cancer. Notably, MMP-2 and MMP-9 may contribute to tumor aggressiveness, not necessarily through direct effects on tumor cell invasiveness but perhaps more indirectly through their effects on other tumor cell properties, such as angiogenesis (55).

The acquisition of invasive potential by tumor cells is undoubtedly a complex process. Our results from in vivo and in vitro studies show that enhanced MT1-MMP expression generally correlates with the invasive potential of cervical cancer and HPV-immortalized keratinocytes but not in squamous epithelial cells derived from LSILs. Given that invasion requires the coordinated expression of cell adhesion molecules, motility machinery, and cell shape changes as the carcinoma cells transit between the two-dimensional and three-dimensional ECM, it is not surprising that other factors in addition to MT1-MMP are likely necessary for squamous epithelial cells to traverse the underlying stroma. Further studies of gene expression in preinvasive and invasive cervical cancers should support with identification of additional factors that mediate tumor cell invasiveness.

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References


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