Down-regulation of MT1-MMP expression suppresses tumor cell invasion in metastatic human SW626 ovarian cancer cells

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Abstract. Membrane-type 1 matrix metalloproteinase (MT1-MMP/MMP-14) is a key enzyme involved in degradation of extracellular matrix (ECM) and various surface-associated proteins that control cell growth, differentiation and survival, plays crucial roles in molecular carcinogenesis, tumor cell growth, invasion, and angiogenesis. We tested the inhibitory effect of antisense MT1-MMP on the ability of metastatic human ovarian carcinoma cell line SW626 in proliferation and invasion. RT-PCR was used to amplify MT1-MMP cDNA fragments with two different restriction sites at its 5'-end. Antisense MT1-MMP cloned in eukaryotic expression vector pMMP14as was transfected into SW626 cells. MT1-MMP protein expression, activities of MMP-2 and MMP-9, changes of cell proliferation, and cell invasion ability were detected by Western blot, optimized gelatin zymography, MTT assay and matrigel *in vitro* invasion assay, respectively. After 48 h transfection, decreased expression of endogenous MT1-MMP protein was detected in pMMP14as-transfected SW626 cells and showed significantly lower proliferation level when compared with control cells. The activation of proMMP-2 was inhibited markedly, and the mean percentage of invasive cells was 63.30±5.80% in pMMP14as-transfected cells, which was less than that (97.60±7.50%) in control cells ($P<0.05$). Both cell proliferation and invasion in SW626 cells were inhibited effectively by antisense MT1-MMP transfection, suggesting that MT1-MMP may be a proper target molecule for anti-invasion therapy for human ovarian cancers.

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Abbreviations: MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; TIMPs, metalloproteinases

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Introduction

Ovarian cancer is the fifth leading cause of cancer death in women and the most lethal gynecologic cancer (1), whereas patient mortality is mainly caused by the metastatic spread of ovarian cancer cells (1). To achieve metastasis, tumor cells must disrupt cell-cell and cell-extracellular matrix (ECM) contacts, and then migrate through stromal tissue, invade the basement membrane, and finally enter/exit the blood stream (2). The degradation of extracellular matrix is accomplished by the matrix metalloproteinase (MMP) enzyme family, such as MMP-2, MMP-9, MMP-7 and MT1-MMP (3,4). MMPs are a family of zinc-binding endopeptidases that collectively degrade most of the components of the extracellular matrix (ECM) (4,5). Elevated level of MMPs has been correlated with invasiveness of several human cancers, such as gastric (6,7), colon (8), and breast carcinomas (9,10), as well as with some tumor phenotypes, such as angiogenesis, cell proliferation, and resistance to apoptosis (3,11). Most MMPs are secreted from cells as inactive zymogens (proMMPs) and are activated extracellularly by serine proteases or other MMPs. In addition to proteolytic activation, normal MMP activity is repressed by tissue inhibitors, metalloproteinases (TIMPs), through activating site inhibition (12).

MMPs can be subdivided into two groups, secreted MMPs and membrane-type MMPs (MT-MMPs). MT-MMPs possess the common domain structure with pre-, pro-, catalytic and hemopexin-like domains of MMP family, and an additional membrane-anchoring domain (4,5,13,14). It is known that the enhanced expression of secreted MMPs, such as MMP-2 and MMP-9, is correlated with the malignant phenotype of human cancer cells both *in vivo* and *in vitro*. However, recent studies demonstrated that membrane-type MMPs, rather than secreted MMPs, are ideally positioned for regulating pericellular proteolysis (4,5). All of the six MT-MMPs (MT1, MT2, MT3, MT4, MT5, and MT6) are able to degrade ECM components, disrupt cell-ECM contacts, and activate proMMP-2, either directly or indirectly via other MMPs, such as MMP-2 or MMP-13 (15-18,21,22). Except these functions, MT1-MMP localize to the leading edge of invasive cells. Interestingly, tissue inhibitor of metalloproteinase-2

(TIMP-2) plays a role in the activation pro-MMP-2 by MT1-MMP, which requires forming of a ternary complex on the cell surface at a specific stiochiometric ratio (13,15,19,20). MT1-MMP is up-regulated in malignant tumors of different tumor cell types such as gastric, colon, and breast carcinomas, and its up-regulation has been implicated in tumor progression and metastasis (4,5,23,24). Over-expression of MT1-MMP in tumor cells was shown to enhance tumor growth and metastatic spread (13,17,25,26). However, it is unclear whether the endogenous expression levels of MT1-MMP in human ovarian cancer cells are correlative with tumor growth and metastasis in presence of other MMPs.

In this study, we investigated the effects of endogenous MT1-MMP expression on metastatic human SW626 ovarian cancer cells in tumor cell proliferation and invasion. Anti-sense MT1-MMP was expressed in SW626 cells by liposome-mediated gene transfer system. Endogenous MT1-MMP expression was successfully down-regulated at an inhibition rate of 65.83%. The endogenous expression of MT1-MMP, tumor proliferation and invasion activity were examined to investigate the effect of anti-sense MT1-MMP transfer on malignant phenotype. Our data suggested that MT1-MMP was essential for the activation of proMMP2, cell proliferation, and invasion into matrigel. Therefore, our results indicate MT1-MMP as a special target for anti-invasion therapy of human ovarian cancers.

Materials and methods

Cell culture and construction of plasmids. The human ovarian cancer cell line SW626 was obtained from American Type Culture Collection (ATCC). SW626 was verified as a cell line with high metastasis potential in our laboratory (27) and maintained in a medium containing DMEM supplemented with 10% fetal bovine-serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

Total RNA of SW626 cells was extracted by using TriZol reagent according to the manufacturer's protocol. cDNA was synthesized by using 5 μg total RNA with Moloney murine leukemia virus reverse transcriptase (M-MLV RT) following the manufacturer's instructions. MT1-MMP was amplified by using sense and antisense primers with different restriction sites, *Eco*RI and *Bam*HI at its 5' end. The sense primer sequence for MT1-MMP was 5'-ccggaattcAAGTTCAGTGCCTACCG AAG-3' and the antisense primer sequence was 5'-cgcggatcc CTTGTCTGGAACACCACATC-3' (lowercases indicate the specific restriction sites). 407 base pair fragment of the PCR products was completely digested with *Eco*RI and *Bam*HI and purified by using the Glassmilk DNA purification kit. And then the fragment was cloned into pcDNA 3.1(+) *Eco*RI and *Bam*HI sites. The ligation product was transformed into *E. coli* $DH_5\alpha$, and the recombinant positive clones, pMMP14as were obtained, and verified by digestion and sequencing.

Cell transfection. For transfection, human ovarian cancer cells SW626 were seeded in six-well plates at $1.5x10^5$/well and incubated in 5% CO_2/95% air incubator at 37˚C. Lipofectamine 2000 was used to increase uptake of plasmid DNA as described by the manufacturer's instruction. Cells were 80-90% confluent on the day of cell transfection experiment. Recombinant plasmid pMMP14as (2 μg) was mixed with lipofectamine and pre-incubated for 15 min at room temperature in serum-free and antibiotic-free DMEM. SW626 cells were treated in a CO_2 incubator at 37˚C until the assay (24-48 h post-transfection). Equal amount of pcDNA 3.1 plasmid was used as a negative control and transfected into SW626 cells. Cells were incubated in the conditioned medium for 12, 24, 36, and 48 h.

Western blotting. Immunoblotting was performed as described previously by Rozanov *et al* (28). Cells harvested at different time points were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P 40, 1% Triton X-100, 0.2% SDS, 1% sodium deoxycholate, 5 mM iodoacetamide, proteinase inhibitor cocktail, 2 mM PMSF). Protein (50 μg) was denatured in SDS sample buffer at 100˚C for 5 min and then separated on a 10% SDS-PAGE gel which was transferred to nitrocellulose membrane by electrophoresis. The membrane was blocked in TBST (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) with 5% non-fat milk for 1 h at 37˚C, and then incubated with the primary antibody against human MT1-MMP at 1:500 dilution in blocking buffer overnight at 4˚C. The membrane was washed three times in TBST and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (1:500) for 2 h at room temperature and then visualized with NBT/BCIP/buffer (1:1:50). As a control for protein loading, nitro-cellulose membranes were reprobed with goat anti-actin serum diluted 1:500 with blocking buffer.

Modified gelatin zymography. Metalloproteinase activity was analyzed in substrate gel zymography, as described previously (29). The conditioned medium at different time points was precipitated with acetone and resolved in 40 mM Tris-HCl buffer. Equal amount of proteins was separated in 8% SDS-PAGE gel co-polymerized with 0.2% gelatin at 4˚C. The gel was rinsed twice in 2.5% Triton X-100 for 15 min at room temperature and then incubated in reaction buffer containing 50 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$ at 37˚C for 10 h. The gel was stained with 0.5% Coomassie blue R250 and destained in 50% methanol and 10% acetic acid.

Analysis of cellular invasion. *In vitro* invasion assay was performed according to a protocol described by Graves *et al* (30). Cellular invasion was measured in 24 trans-well units with 8 μm porosity polycarbonate filters coated with 50 μl reconstituted basement membrane substance matrigel. Tumor cells ($2.5x10^4$) were placed in the upper chamber of the trans-well unit. The lower chambers were filled with serum-free conditioned medium of NIH 3T3. After 10 h incubation at 37˚C in a humidified 95% air and 5% CO_2 atmosphere, non-invasive cells were removed from the upper surface of the membrane with a cotton swap, and then cells were fixed with 70% methanol at room temperature for 45 min and stained with hematoxylin. Cell invasion processes were measured by counting the cells that migrated to the lower side of the polycarbonate filters (cell numbers in six random areas were counted under light microscope, x10). All the experiments were repeated at least three times.

Statistical analysis. The data are expressed as mean ± SD. P<0.05 was considered statistically significant. Statistical analysis was done by using SPSS 10.0 for Windows by Student's t-test.

Results

Construction of the recombinant plasmid pMMP14as. An active form of MMP-2 has frequently been detected in tumor tissues (4,13). The expression of MT1-MMP has been demonstrated in tumor tissues in which MMP-2 is always activated (4,7,8). We found increased expression of MT1-MMP in human ovarian cancer cells SW626 with high metastasis potential and then detected the 62-kDa active form of MMP-2 in cell conditioned medium. To confirm that the activation ratio of MMP-2 correlates with MT1-MMP expression level, antisense MT1-MMP eukaryotic expression vector, pMMP14as, is constructed and expressed in SW626 cells (Fig. 1A). These results indicated that antisense recombinant plasmid pMMP14as verified by digestion and sequencing had successfully been constructed.

Effect of pMMP14as on activation of ProMMP2 in SW626 cells. It was reported that MT1-MMP is able to activate proMMP-2 when MT1-MMP was over-expressed in HT1080 cells (31). However, it is not clear whether MT1-MMP has the same effect in activating proMMP-2 in SW626 cells. To examine how MT1-MMP contributes to the activation of proMMP-2, antisense MT1-MMP eukaryotic expression vector, pMMP14as was transfected into the SW626 cells. The endogenous MT1-MMP expression was decreased while the actin expression was not affected. After 36 h treatment, the inhibitory effect on MT1-MMP expression was increased and maximal at 48 h. The inhibitory rate reached 65.83% (Fig. 1B), demonstrating that decreased expression of endogenous MT1-MMP in SW626 cells was a result of transient transfection of pMMP14as. The inhibition of matrix metalloproteinase activity was confirmed by modified gelatin zymography. Three gelatinolytic bands at 92 kDa, 72 kDa and 62 kDa, corresponding to the latent form of MMP-9 and the latent and active forms of MMP-2, respectively, were detected in a conditioned medium in SW626 cells. The cells transfected with pMMP14as showed gradually reduced activation of MMP-2, which was indicated by the intensity of the gelatinolytic bands at 62 kDa. Section of proMMP-9 and its activation were not affected by the pMMP14as (Fig. 1C). Our study demonstrated that MT1-MMP is the major activator of proMMP2 in SW626 cells.

Effect of pMMP14as on the cell proliferation and invasion. Uncontrolled proliferation of tumor cells is one of the most important characteristics of malignancy (32). SW626 cells are strongly tumorigenic in ovarian cancer orthotopic transplantation model. Thus, we investigated the effect of various expression of endogenous MT1-MMP on cell proliferation. At 36 h post-transfection, there was no difference in cell proliferation in the pMMP14as-transfected group compared to the pcDNA3.1-treated control group. After 48 h, the cell growth in the pMMP14as-transfected group was significantly slower than that transfected with pcDNA3.1 only (Fig. 2A).

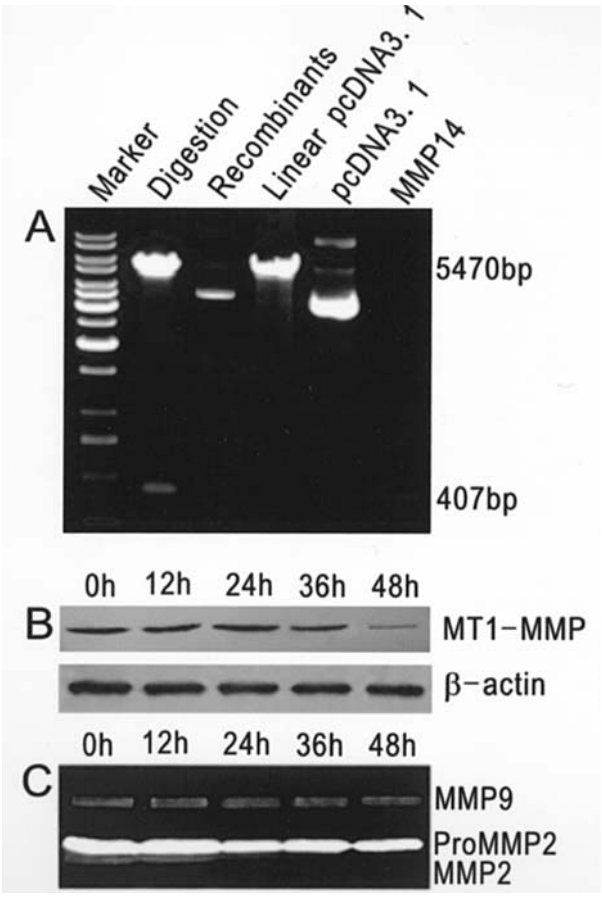

Figure 1. Construction of antisense MT1-MMP eukaryotic expression vector pMMP14as and the effect of special downregulation of MT1-MMP expression on the activation of proMMP-2 in SW626 cell. A, Identification of recombinant plasmid pMMP14as by restriction enzyme digestion. (lane 1, 1 kb DL-2000 marker; lane 2, pMMP14as digestion; lane 3, pMMP14as recombinant plasmid; lane 4, pcDNA3.1 digestion; lane 5, pcDNA3.1; lane 6, 407 bp MT1-MMP PCR products). In lane 2, a 407 bp band indicates successful construction of pMMP14as. B, Special downregulation of MT1-MMP expression using transient tranfection of pMMP14as into SW626 cells. Western blot was performed to test MT1-MMP protein level in SW626 cells transfected with pMMP14as by using anti-MT1-MMP monoclonal antibody. ß-actin was used to demonstrate equal loading. C, The pMMP14as-suppressed activation of proMMP-2 in SW626 cells. SW626 cells were transfected with pMMP14as. After transfection, the conditioned medium collected on the indicated hour was analysed by modified gelatin zymography. Pro-, and active forms of MMP-2 and MMP-9 are indicated.

These data indicated that decreased expression of MT1-MMP significantly inhibited tumor cell proliferation.

Tumor cell invasion requires co-ordination between degradation of the ECM barrier and cell locomotion. SW626 is a well-established invasive cell line in matrigel *in vitro* invasion assay. The cells transfected with pMMPas were grown on matrigel, and the cells that invaded the matrigel were enumerated. The invasive tumor cell percentage (63.30±5.80%) in the pMMP14as-transfected group was lower than that in the pcDNA3.1 or control one (98.10±6.50%) vs. (97.70±7.50%) (P<0.05) (Fig. 2B), suggesting that after transfection with pMMP14as the tumor cell invasiveness decreased significantly, while transfection of the pcDNA3.1 empty vector or control did not affect the cell migration and invasion. These results suggested that MT1-MMP plays a critical role in facilitating SW626 cells invasion.

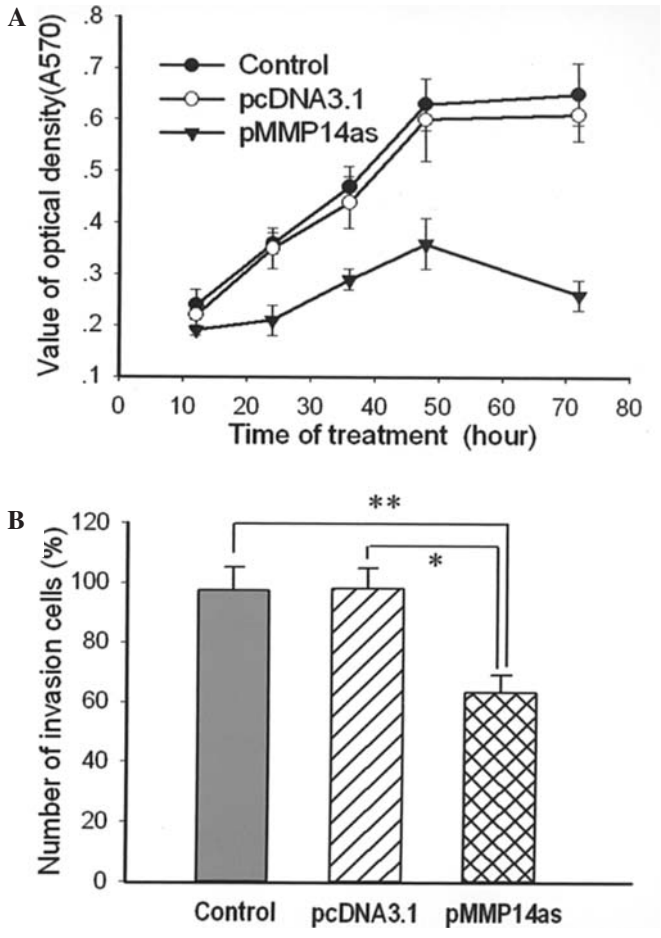

Figure 2. Effect of pMMP14as on tumor cell proliferation and invasion of SW626 cells. SW626 cells were transfected with pMMP14as or pcDNA3.1 empty plasmid. A, The impact of pMMP14as on SW626 ovarian cancer cell proliferation. Equal numbers ($5.0x10^3$ cells) of SW626 cells was seeded in 96-well plates. After 24 h, 80% confluent SW626 cells were transfected with pMMP14as or pcDNA3.1 empty plasmid. After transfection, proliferation of pMMP14as-transfected cells and pcDNA3.1 transfected cells was monitored over a period of 72 h, starting 12 h after transfection. The absorbable values in pMMP14as-transfected cell were measured at 570 nm wavelength. Values and error bars in this graph represent the averages and standard deviations of three independent experiments. B, *In vitro* invasion assays were carried out in the matrigel-coated membrane inserts. Non-coated membrane inserts were seeded to serve as control. Each assay was performed in triplicate. Data were expressed as percentage of invasion: the ratio of cells invading through the matrigel coated inserts compared to uncoated control inserts. The levels of significance: $^*P=0.002$; $^{**}P=0.003$.

Discussion

Normal ovarian epithelial cells do not express MMPs, and acquisition of their expression by ovarian carcinomas may promote more aggressive invasion (33,34). MMPs associated with ovarian carcinomas include MMP-2 (M_r 72000 gelatinase A), MMP-9 (M_r 92000 gelatinase B), and the cell membrane-anchored MMP, MT1-MMP. In this study, we found that the expression level of MT1-MMP was increased in human ovarian cancer cells SW626 with high metastasis potential and then the 62-kDa active form of MMP-2 was detected in SW626 cell conditioned medium. Recent observations indicated that the expression level of MT1-MMP, the most important one of MT-MMPs, is up-regulated in most invasive tumors (7-10). As MT1-MMP localizes on lamellar pseudopodia of the migration front of the invasive tumor cells, MT1-MMP not only degrades ECM molecules, such as collagen types I, II, and III, fibronectin, laminin-1 and -5, etc, but also participates in the activation process of matrix metalloproteinase groups by recruiting proMMP-2 to the cell surface and causes its activation as well as up-regulating VEGF to improve tumor growth and angiogenesis (4,31,35). Therefore, MT1-MMP, when overexpressed, strongly promotes cellular invasion *in vitro* and experimental metastasis (17,36). To further elucidate the role of endogenous MT1-MMP expression in the tumor invasiveness, we successfully constructed antisense MT1-MMP eukaryotic expression vector.

The antisense nucleotide technique, which can selectively shut off and modify the special gene in transcription level (37), was successfully applied to this study that the expression of MT1-MMP was suppressed in SW626 cells. Transient transfection of pMMP14as construct against MT1-MMP suppressed the expression to 17% at 36-48 h at the protein level. It was reported that all of MT-MMP were expressed in HT1080 cells and MT1-MMP played a major role in the activation of proMMP-2 in HT1080 cells. However, it is still not clear whether MT1-MMP is the major player of the activation of proMMP-2 in SW626 cells. Down-regulation the expression level of MT1-MMP clearly showed its predominant role in the activation of proMMP-2 in SW626 cells. This is consistent with previous observations that tissue extracts from MT1-MMP-null mice exhibited decreased activation of proMMP-2, indicating that MT1-MMP is the major activator of proMMP-2 in mice (38). However, it is still possible that proMMP-2 activation by other MT-MMPs occurs in some tissues and situations. Our data indicated that in tumor tissues and tumor cell lines, the expression levels of MT1-MMP correlate well with the activation of proMMP-2.

Tumor cell invasion requires a concerted organization of ECM degradation and cell locomotion in the matrix. The expression level of MT1-MMP was increased in SW626 cells, suggesting the expression level of MT1-MMP correlated positively with its metastasis potential. Although MMPs are collectively important for the degradation of ECM, down-regulation of MT1-MMP was sufficient to inhibit the invasion of SW626 cells in the matrigel. Our data indicated that, after 48 h transfection with pMMP14as, the growth rate was inhibited to a certain extent and the tumor cell invasiveness was decreased significantly. Although it has been described earlier that MT1-MMP specially localized to tumor cells in invasive ovarian cancer, our study is the first demonstration that MT1-MMP is a potentially effective target to treat invasive ovarian tumors.

Despite enthusiasm for MMP inhibitors, phase III trials have not yet demonstrated clearly benefit in overall survival, and side-effects of the inhibitors may remain a problem (39). The broad-spectrum MMP inhibitors may suppress the host defense system and diminish the therapeutic effect, because MMPs are not only important for cancer growth and invasion, but also for the host defense (40). Our observations raised a possibility that decreasing a single MMP, MT1-MMP, gene expression is able to suppress the invasive property of aggressive tumors. If inhibition of MT1-MMP does not affect the host defense system significantly, specific

targeting of MT1-MMP would have a better therapeutic effect on human invasive ovarian cancer.

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