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Relationship between expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 and invasion ability of cervical cancer cells

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Abstract. Constitutive overexpression of matrix metalloproteinases (MMPs) is frequently observed in malignant tumors. MMPs are a family of zinc endopeptidases consisting of at least 20 different members. In particular, MMP-2 and MMP-9 are reported to be closely associated with invasion and metastasis in several cancers. We investigated whether expression of MMP-2 and MMP-9 is associated with invasion ability of seven cervical cancer cells by administration of o-phenanthroline as MMP inhibitor. In two cell lines, Siha and Caski, *MMP-2* mRNA and protein were expressed at high levels. After treatment with o-phenanthroline, the rate of invasion in these two cell lines was significantly decreased. In contrast, in the other two cell lines, HT-3 and Caski, high levels of *MMP-9* mRNA and protein were expressed but there was no decrease in the rate of invasion in these cells after treatment with o-phenanthroline. The data suggest that expression level of *MMP-2* mRNA may regulate with invasion ability of cervical cancer.

Introduction

Degradation of the extracellular matrix (ECM) surrounding a tumor cell is an essential step for invasion and metastasis of malignant cells (1,2). Matrix metalloproteinases (MMPs) that degrade various ECM components are frequently expressed in malignant tissues at higher levels than in their normal counterparts (3,4). MMPs are a family of zinc endopeptidases consisting of at least 20 different members. They are classified into five groups based on their structure and substrate specificities (5,6). MMPs are secreted from cells as pro-enzymes and their synthesis is regulated by hormones, growth factors, and cytokines (7-9). In addition, MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The activities of MMPs can be inhibited *in vitro* by metal chelators such as o-phenanthroline and EDTA (6,10,11), because zinc and calcium ions are essential for stability of the enzyme. Among the MMPs, MMP-2 and MMP-9 are secreted as progelatinase-A and progelatinase-B, respectively, to degrade type IV collagen and they are thought to play an important role in the invasion of the cancer cells (12-15). However, little is known about the relationships among invasion ability, expression, and production of MMP-2 and MMP-9 by cervical cancer cells. In this study, in order to understand the roles of MMP-2 and MMP-9 in the pathologic process of cervical cancer, we investigated mRNA expression levels of *MMP-2* and *MMP-9* and the invasion ability of cervical cancer cell lines. Messenger RNA expression of *MMP-2* and *MMP-9* was measured using real-time PCR, and then gelatin zymography and invasion assay by administration of o-phenanthroline as MMP inhibitor were performed.

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Key words: cervical cancer, MMP-2, MMP-9, MMP inhibitor, real-time PCR, zymography, invasion assay

Materials and methods

Cervical cancer cell lines and cell culture. Seven human cervical cancer cell lines (HT-3, Siha, ME-180, Hela-S3, Caski, C-4I, and C-33A) were obtained from the American Type Culture Collection (Rockville, MD, USA). They were maintained in Dulbecco's modified Eagle medium (D-MEM, low glucose; Nikken Bio Medical Laboratory, Japan) supplemented with 10% fetal bovine serum (Gibco BRL, MD USA), penicillin (100 unit/ml) and streptomycin (100 mg/ml) at 37˚C in tissue culture flasks (Iwaki, Japan) in a humidified incubator gassed with 5% CO_2.

RNA preparation and reverse transcription. Confluent cell layers were washed twice with phosphate-buffered saline (PBS) before harvesting. Total RNA was isolated from each cell line using Isogen (Nippon Gene, Japan). Prior to reverse transcription, total RNA was treated with DNase I at 37˚C for 10 min to remove contaminated genomic DNA. Each 20 μl of reaction mixture contained 1 µg of total RNA, 4 µl supplied PCR buffer (50 mM Tris-HCl; pH 8.3; 75 mM KCl; 3 mM magunesium chloride, Gibco BRL, MD, USA), 0.5 mM of dNTP (Takara, Japan), 200 units of moloney murine leukemia

Table I. PCR primers of *MMP-2*, *MMP-9* and *ß-actin*.

	Sense	Antisense	Target size (bp)
MMP-2	5'-caaggagtacaacagctgcactgata-3'	5'-ggtgcagctctctcatatttgttgc-3'	369
MMP-9	5'-tgggcaagggcgtcgtggttc-3'	5'-tggtgcaggcggagtaggatt-3'	276
ß-actin	5'-gtttgagaccttcaacacccc-3'	5'-gtggccatctcttgctcgaagtc-3'	318

virus reverse transcriptase (Gibco BRL, MD, USA), and 1 µl of oligo(dT)$_{12-18}$ primer (0.5 mg/ml) (Gibco BRL, MD, USA). Reverse transcription was carried out at 37˚C for 60 min and followed by 5 min at 95˚C to terminate the reaction.

Polymerase chain reaction (PCR). The mixture of reverse transcriptant was subjected to PCR. Each volume (25 µl) contained 1 µl of RT reaction product as template DNA, 12.5 µl of HotStart Taq Master Mix (Qiagen Inc., CA, USA), and 20 pmol each of sense and antisense primers of the target genes (Table I). PCR was performed with the first cycle at 95˚C for 15 min to activate HotStar Taq polymerase in the reactant, followed by 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. For the amplifications of *MMP-2*, *MMP-9*, and ß-actin, there were 35, 35, and 30 cycles respectively. PCR products were then electrophoresed through 1.5% agarose gels stained with ethidium bromide. PCR products were visualized with UV light.

Real-time PCR. To confirm mRNA expression of *MMP-2* and *MMP-9* and to evaluate their mRNA expression levels in the cultured cells in this study, real-time PCR was applied using *MMP-2* and *MMP-9* specific primers (Table I) with Smart-Cycler™ (Cepheid, CA, USA). Each 25 µl of PCR mixture contained 0.3 µM of gene specific primers, 1 µl of sample cDNA solution, 12.5 µl of PCR premixture (Quantiteck SYBR Green PCR Kit™, Qiagen Inc., CA, USA) and 6.5 µl of supplied water. PCR was performed with the first cycle at 95˚C for 15 min to activate HotStar Taq polymerase in the premixture, this was followed by 45 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. The signal intensity was measured at 72˚C during each cycle. The absence of bands other than the target band was verified by gel electrophoresis on 1.5% agarose stained with ethidium bromide under UV light after amplifications. For quantitative analysis of *MMP-2*, *MMP-9* and *ß-actin* expression, standard DNA solutions were produced by serial dilutions of column-purified PCR products (PCR purification Kit, Qiagen Inc., CA, USA) showing the target band. The original PCR product containing 25 ng/µl of target DNA was diluted into concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} with distilled water, and these dilutions were simultaneously subjected to real-time PCR to establish a standard curve. To adjust the difference of concentration of mRNA reverse-transcribed, *ß-actin* was used as an internal control. The procedure for measurement of *ß-actin* mRNA expression was the same as that for *MMP-2* and *MMP-9* mRNA expression described above. Relative quantity was evaluated by ratio of mRNA expression of the target gene and *ß-actin*.

Gelatin zymography. Gelatin-substrate gel electrophoresis was used to measure the levels of metalloproteinase activity in samples from cell lines (gelatin zymography kit, Yagai Corporation, Japan). Cervical cancer cell lines were seeded onto plates in D-MEM containing 10% FBS. When the cells had grown to approximately 80% confluency, the medium was removed, and the cells were washed 3 times with D-MEM to remove residual FBS. Cells were then cultured for 72 h in D-MEM with 0.1% BSA. After 72 h, the culture medium was collected, and centrifuged twice at 800 rpm for 5 min. Twenty µl of supernant was electrophoresed on a supplied gel of the gelatin zymography kit. The gel was washed with two kinds of washing buffer, each for 30 min, and then incubated for 30 h at 37˚C in the reaction buffer. Reaction buffer was mixed at 0, 5, or 50 µM o-phenanthroline. Gels were stained with Coomassie blue and de-stained. Gelatinolytic activity was visualized as clear white bands against a blue background.

Invasion assay. The invasion assay was performed by the method of Albini *et al* with modification (16). Cells were cultured in 24-well plates. Into each well of the plate an insert was placed, effectively creating a lower chamber and upper chamber. The bottom of the insert was made of 8.0 µm pore size polycarbonate membranes which were either coated with Matrigel or without any coating (Becton Dickinson BioCoat, NJ, USA). The lower chamber was filled with 700 µl of D-MEM supplemented with 0.1% BSA culture medium of NIH3T3 cells which acted as chemoattractants for the cancer cell lines. Cells grown as subconfluent were harvested and suspended in 500 µl of D-MEM supplemented with 0.1% BSA and 0, 5, or 50 µM of o-phenanthroline. The cells were then cultured at a density of 5.0x10^{4} cells/well in the upper chamber. Each experiment was done in triplicate wells. After incubation for 72 h, the cells on the upper surface of the filters were completely removed by wiping with cotton swabs. The filters were fixed in 70% ethanol and Giemsa stained. The cells on lower surface were counted at x200 magnification in 5 randomized field views. Percentages of invading cells were calculated by dividing the number of cells in matrigel by the number of invading cells in the non-matrigel. Mean percentages were obtained from results from the triplicate wells.

Statistical analysis. The data of rate of invasion were analyzed using the Student's t-test. Regression analysis was performed for the study on relationship between difference in the rate of invasion and the mRNA expression levels of *MMP-2* and *MMP-9*. P-value of less than 5% was considered significant.

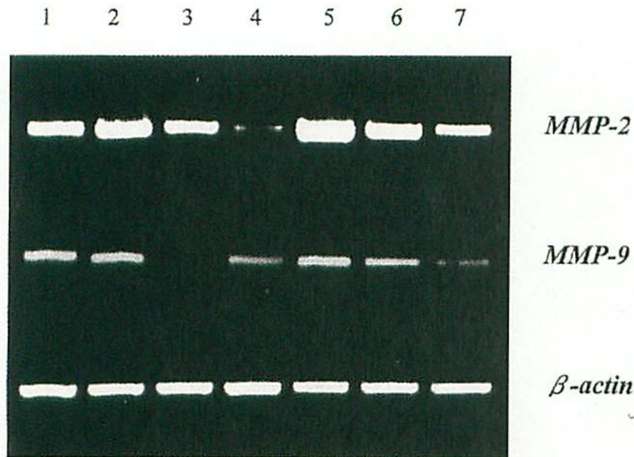

Figure 1. Messenger RNA expression of *MMP-2*, *MMP-9* and *β-actin* using RT-PCR in seven cervical cancer cell lines. *MMP-2* (upper panel), *MMP-9* (middle panel), *β-actin* (lower panel). Lane 1, HT-3; lane 2, Siha; lane 3, Hela-S3; lane 4, ME-180; lane 5, Caski; lane 6, C-4I; lane 7, C-33A. *MMP-2* and *MMP-9* were expressed in all the cell lines.

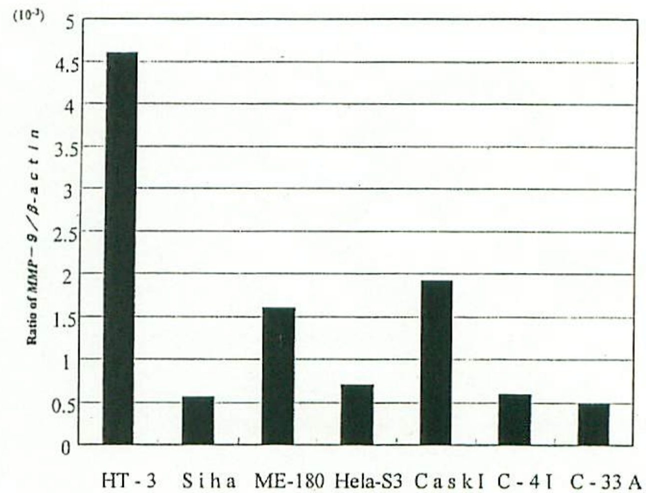

Figure 3. The expression ratio of *MMP-9/β-actin* in seven cervical cancer cell lines.

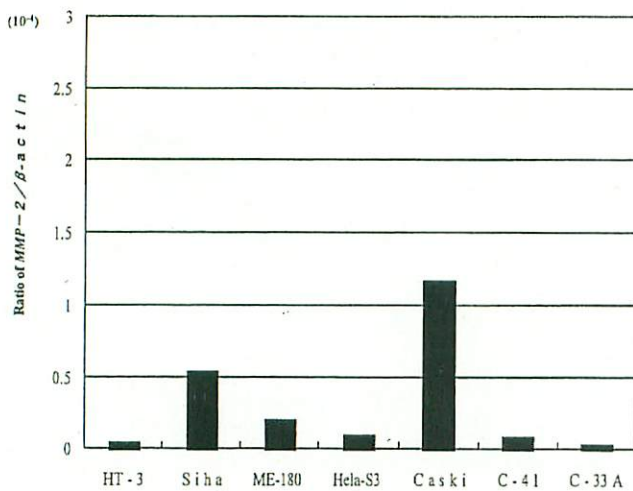

Figure 2. The expression ratio of *MMP-2/β-actin* in seven cervical cancer cell lines.

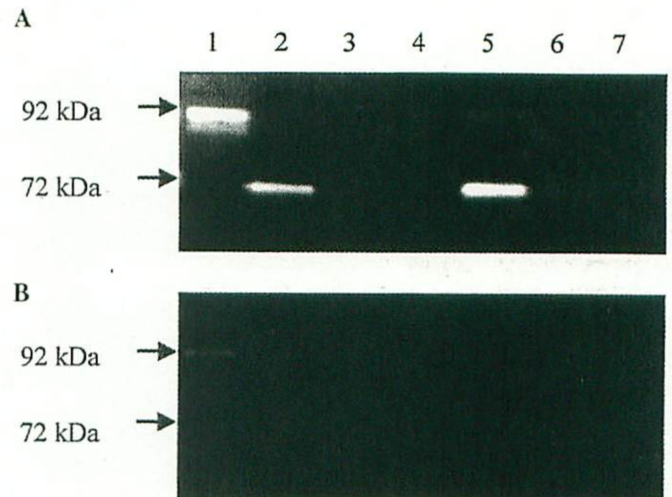

Figure 4. Matrix metalloproteinase (MMP) activity in gelatin zymography. Reactions were carried out in reaction buffer alone (A), and reaction buffer containing o-phenanthroline 5 μM (B). There was no band within reaction buffer containing o-phenanthroline 50 μM (data not shown). Lane 1, HT-3; lane 2, Siha; lane 3, Hela-S3; lane 4, ME-180; lane 5, Caski; lane 6, C-4I; lane 7, C-33A. Siha and Caski secreted progelatinase-A (72 kDa), HT-3 and Caski secreted progelatinase-B (92 kDa) (secreted expression of Caski was very low) (A). HT-3 only secreted progelatinase-B (B). All experiments were repeated three times with identical results.

Results

PCR analysis. Messenger RNA expression of *MMP-2*, *MMP-9* and *β-actin* was detected in all of seven cervical cancer cells by RT-PCR. Messenger RNA expression of *MMP-2* was high in Siha and Caski, but low in ME-180. Messenger RNA expression of *MMP-9* was high in HT-3 and Siha and Caski but weak in Hela-S3 and C-33A. No difference was found in the mRNA expression level of *β-actin* among the cell lines (Fig. 1).

According to the results of real-time PCR, the ratios of *MMP-2/β-actin* in Caski and Siha were about 20 and 10 times, respectively, the ratio of *MMP-2/β-actin* in HT-3 that had the lowest ratio among the cell lines (Fig. 2).

The ratio of *MMP-9/β-actin* was lowest in Siha. In HT-3 and Caski, ratios of *MMP-9/β-actin* were high, 8.07 and 3.33 times of that of Siha (Fig. 3).

Zymography. The supernatants were assayed for MMP-2 and MMP-9 activity by the gelatin zymography and reactions were carried out in reaction buffer containing o-phenanthroline at 0, 5, and 50 μM, respectively.

In zymography, o-phenanthroline was not detected; progelatinase-A and progelatinase-B were detected as bands of 72 and 92 kDa, respectively. Progelatinase-A was expressed in Siha and Caski, and expression was stronger in the latter. Progelatinase-B was detectable in HT-3 and Caski, and the expression was stronger in HT-3 (Fig. 4A). When o-phenanthroline was added to the reaction buffer at 5 μM, expression of the two progelatinases became weak, only progelatinase-B in HT-3 was detectable (Fig. 4B).

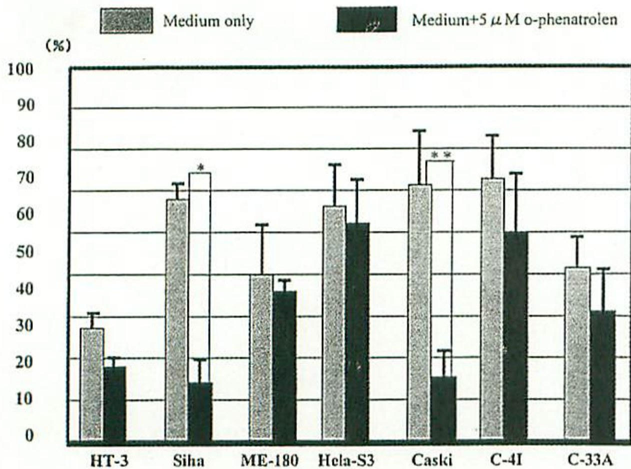

Figure 5. Invasion assay. In the presence of o-phenantroline, invasion ability of all the cell lines was weakened. The difference in Siha and Caski was statistically significant (*p=0.0006, **p=0.0069). The data are shown as mean value ± SD of three different experiments.

The progelatinases were not detectable in any cell lines when o-phenanthroline was at 50 μM (data not shown).

Invasion assay. The amount of o-phenanthroline (0, 5 and 50 μM) was determined according to the results of zymography. When o-phenanthroline was not present, no obvious difference in the invasion rate between the cell lines was found. However, invasion rates of Siha and Caski decreased significantly at 5 μM o-phenanthroline (Siha, from 65.6 to 16%; Caski, from 69.9 to 18%). There was no significant decrease rate in the other five cell lines (Fig. 5). At 50 μM o-phenanthroline of solution, because the rate of cell growth was low, the analysis of invasion assay could not be completed after 72 h (data not shown).

Relationship between mRNA expression level of MMP-2 and MMP-9 and the invasion ability of the cancer cells. We further investigated whether the difference in rate of invasion was related to the mRNA expression levels of *MMP-2* and *MMP-9* in all cell lines. Regression analysis revealed that the difference in rate of invasion and the mRNA expression level were significant in *MMP-2* (r=0.842, p=0.039), but not in *MMP-9* (r=-0.188, p=0.716).

Discussion

Degradation of the surrounding ECM is thought to be one of the important initial steps in the complex processes of tumor invasion (1). This step depends in great part on the appropriate proteolytic enzymes, such as MMP family, that digest the complex barrier. The MMP family consists of at least 20 members (6), each of which contains a zinc ion. MMPs are secreted as proenzymes that are activated after a peptide of about 10 kDa is cleaved. The activity of MMPs is regulated at the level of transcription, translation and secretion. They are inhibited by specific tissue inhibitor matrix metalloproteinases (TIMPs) and metal chelator (1-3). Increased expressions of MMPs have been associated with cancer of the tongue, head and neck, breast, lung, stomach, and pancreas (17-22). In particular, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), which degrade type IV collagen, were reported to correlate with tumor grade and metastasis (15,23). However, there are few reports addressing the role of MMPs in gynecologic cancers (24-26). In this study, we investigated expression of MMP-2 and MMP-9 in cervical cancer, and its relationship to invasion ability by seven cervical cancer cell lines. In analysis of MMPs, observation of mRNA expression level and measurement of proteinase activity are the methods most frequently used. For example, Kitagawa *et al* studied expression of mRNA and its relationship to pathologic stage, pathologic grade, cell type of renal cancer (27). Davies *et al* have used quantitive zymography to measure levels of MMP-2 and MMP-9 in transitional cancer (4). Like studies by Kitagawa *et al* and Davies *et al*, we used real-time PCR and zymograpy, to evaluate expression mRNA and protein of MMP-2 and MMP-9 (4,27). Interestingly, two cell lines that had high mRNA expression levels of *MMP-2* and *MMP-9* were found on zymography. Therefore, the mRNA expression level of the genes was in accord with the expression level of protein in these cell lines. To investigate whether the mRNA expression levels of *MMP-2* and *MMP-9* were related to invasion ability, as was expected, their inhibitor, o-phenanthroline was used in the invasion assay. We found that the protein expression levels of MMP-2 and MMP-9 were markedly decreased on zymography when 5 μM o-phenanthroline was added to the culture. Consequently, the invasion rate of Siha and Caski was significantly reduced. These results led us to speculate that there was a strong relationship between the mRNA expression level of *MMP-2* and invasion ability of the cells. However, a similar relationship in *MMP-9* was not found. Therefore, MMP-2 may serve as one of tumor invasion pathways in cervical cancer cells.

Surgery, radiotherapy and chemotherapy are still the mainstay of cancer treatments. However, several MMP inhibitors were proposed as potential drugs for treatment of cancer (28-31) and are now being evaluated in phase clinical trial. In this sense, our study may provide important information about the regulation of invasion in cervical cancer cells.

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