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Macrophage Inhibitory Cytokine-1 Induces the Invasiveness of Gastric Cancer Cells by Up-Regulating the Urokinase-type Plasminogen Activator System[1]

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ABSTRACT

In our search for genes associated with gastric cancer progression, we identified macrophage inhibitory cytokine-1 (MIC-1), a member of the transforming growth factor β superfamily, as an overexpressed gene in gastric tumor tissues. Expression analysis of *MIC-1* in gastric tumor tissues revealed a specific expression in gastric cancer cells, and this expression level was well correlated with invasive potential in various human gastric cancer cell lines. Stable transfection of *MIC-1* into SNU-216, a human gastric cancer cell line, significantly increased its invasiveness. The overexpression of *MIC-1* into SNU-216 cells significantly increased the activity of urokinase-type plasminogen activator (uPA), and the expressions of uPA and urokinase-type plasminogen activator receptor (uPAR). Similarly, the stimulation of gastric cancer cell lines with purified recombinant MIC-1 dose-dependently increased cell invasiveness, uPA activity, and uPA and uPAR expression. However, MIC-1 did not significantly suppress the proliferation of gastric cancer cell lines. We also found that the stimulation of human gastric cell lines with recombinant MIC-1 strongly induced activation of mitogen-activated protein kinase kinase-1/2 and extracellular signal-regulated kinase-1/2. Additional analysis revealed that PD98059, a selective inhibitor of mitogen-activated protein kinase kinase-1/2, suppressed not only gastric cancer cell invasiveness and uPA activity, but also the mRNA expressions of uPA and uPAR, as induced by recombinant MIC-1. Our results indicate that MIC-1 may contribute to the malignant progression of gastric cancer cells by inducing tumor cell invasion through the up-regulation of the uPA activation system via extracellular signal-regulated kinase-1/2-dependent pathway.

INTRODUCTION

Since it was first recognized that tumors had the ability to invade adjacent tissues and spread to distant organs, extensive research has been performed, which has enormously expanded the body of knowledge concerning this basic hallmark of malignancy. Such spread and distant settlement of tumor cells is the major cause of human cancer deaths (1). With their abilities for invasion and metastasis, cancer cells are able to escape the primary tumor mass and colonize new body tissues. An essential step in metastatic spread involves the invasion of the ECM[4] and basement membranes by tumor cells, which in turn requires the participation of several proteolytic enzyme systems, which involve the urokinase pathway of plasminogen activation (2, 3). In this system, the uPA is secreted as a single-chain inactive proenzyme, which, on binding to uPAR on the cell surface, is cleaved into the active two-chain molecule. After activation, cell-bound uPA is capable of converting plasminogen into plasmin, which is then able to degrade several components of the ECM and can activate certain growth factors as well as latent metalloproteinases required for invasion (4). It has been observed that the expression levels of uPA and uPAR are well correlated with malignant progression in a variety of cancers (2, 5).

MIC-1 is the first member of a divergent group within the TGF-β superfamily (6, 7). It has also been reported as a placental TGF-β (8), a prostate-derived factor (9), a growth/differentiation factor 15/MIC-1 (10), nonsteroidal anti-inflammatory drugs activated gene-1 (11), and as a placental bone morphogenetic protein (12). As with all of the TGF-β superfamily members, MIC-1 is synthesized as a 308-amino acid polypeptide that encompasses a 29-amino acid signal peptide, a 167-amino acid propeptide, and a 112-amino acid mature region (6). The mature protein is secreted as a disulfide-linked homodimer comprised two 112 amino acid mature regions, which is released from the propeptide after intracellular cleavage at a typical RXXR furin-like cleavage site (6). Importantly, unlike all of the other TGF-superfamily members studied to date, the MIC-1 mature peptide can be correctly folded and secreted without a propeptide (13, 14). The major function of MIC-1 is still uncertain, although it has been described variously as being able to inhibit tumor necrosis factor α production from lipopolysaccharide-stimulated macrophages (6), to induce cartilage formation and the early stages of endochonadal bone formation (9), and to inhibit the proliferation of primitive hemopoietic progenitors (12). Several reports have shown that the MIC-1 promoter region is a target for the p53 tumor suppressor gene product (15–18) and that it can also suppress tumor cell growth *in vitro*, although very high concentrations of MIC-1 were required to elicit this effect (15). The very high level of MIC-1 mRNA in the human placenta suggests that MIC-1 may be important for placental function and/or fetal development (19). Recent findings that MIC-1 is highly and specifically expressed in prostate (20, 21) and colorectal cancer tissues (22) suggest its role in tumor development or progression.

In present study, we found that MIC-1 is specifically expressed in gastric tumor tissues and investigated the possible role of MIC-1 in gastric cancer progression.

MATERIALS AND METHODS

Cell Culture. The human gastric cancer cell lines SNU-1, -16, -216, -484, -620, and -638 were obtained from Korean Cell Line Bank of the Cancer Research Center, Seoul National University College of Medicine (Seoul, Republic of Korea), and maintained in RPMI 1640 (Invitrogen Corporation, Carlsbad, CA). The characteristics of these cell lines were described previously (23, 24). Human embryonic kidney cell line 293 and Phoenix cells were maintained in DMEM (Invitrogen Corporation). All of the media were supplemented with 10% fetal bovine serum, penicillin, and streptomycin, and all of the cell lines were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Construction of MIC-1 Expression Vectors. The complete coding region of human MIC-1 cDNA was amplified from SNU-638 cDNA library by PCR

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[4] The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; MIC, macrophage inhibitory cytokine; TGF, transforming growth factor; PAI, plasminogen activator inhibitor; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; MMP, matrix metalloproteinase.

with the following primers: sense primer 5′-CG*GAATTC*ATGCCCGGGCAAGAACTC-3′ (inserted *Eco*RI site italicized) and antisense primer 5′-CC*CTCGAG*TCATATGCAGTGGCAGTC-3′ (inserted *Xho*I site italicized). The amplified cDNA was subcloned into *Eco*RI and *Xho*I site of pBluescript KS (Stratagene, La Jolla, CA), sequenced, and subcloned into pLXSN (Clontech, Palo Alto, CA) to make pLXSN-MIC-1. To construct an expression plasmid for recombinant MIC-1, the region of mature MIC-1 coding amino acid 197–308 (6) was amplified with *pfu* polymerase (Stratagene), and then subcloned with in frame into *Eco*RI and *Xho*I site of pFLAG-CMV-1 (Sigma, St. Louis, MO), which had a NH_2-terminal FLAG tag, and designated as pFLAG-MIC-1.

Preparation of Recombinant MIC-1. Human embryonic kidney cell line 293 cells were transfected with pFLAG-MIC-1 in 10 150-mm dishes. Three days after transfection by calcium phosphate method, the cells were washed three times with PBS, added 10 ml of lysis buffer [50 mM Tris-HCl, 150 mM NaCl (pH 7.4,) 1 mM EDTA, and 1% Triton X-100], and incubated the cells for 30 min. The cells were collected with a scrapper and centrifuged to remove insoluble materials for 10 min at 12,000 rpm. The resulting supernatant was loaded on anti-FLAG M2 affinity column (Sigma), and the column was washed with Tris-buffered saline [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)]. The bound recombinant MIC-1 was eluted with 100 mM glycine (pH 3.0) in stepwise manner and collected in 1.5 ml tube containing 50 μl of 1 M Tris-HCl (pH 8.0). The pooled fraction was dialyzed against PBS and stored at −70°C until use.

Retroviral Infection of SNU-216 Cells with *MIC-1*. The retrovirus was produced using Phoenix cells. Briefly, the cells were plated in 10-cm culture plates in a density of 2×10^6 cells before transfection. Twenty μg of pLXSN-MIC-1 was used for transfection using calcium phosphate kit according to the manufacturer's instruction (Clontech). The cells were treated with 10 mM sodium butyrate for 12 h, washed with PBS, refed with 10 ml of fresh medium, and harvested the medium containing retrovirus 48 h after incubation at 37°C in 5% CO_2. SNU-216 cells were infected with the culture supernatants containing retrovirus at 37°C in 5% CO_2 for 8 h. The 0.5 mg/ml of G418 was used to select infected cells.

***In Vitro* Migration and Invasion Assays.** The ability of cells to migrate through noncoated (migration) or invade through Matrigel-coated filters (invasion) was determined using a modified 24-well Boyden chamber (Corning Costar, Cambridge, MA; 8-μm pore size) as described previously (25). The cells were seeded at a density of 5×10^4 cells in 100 μl RPMI 1640 containing 0.5% BSA (migration) or RPMI 1640 containing 10% FBS (invasion) in the upper compartment of transwell. To determine the effect of MIC-1, various concentrations of recombinant MIC-1 were added to the lower or upper compartment of transwell in the presence or absence of PD98059 (Calbiochem, La Jolla, CA). In a certain experiment, the conditioned media were added to the lower or upper compartment of the transwell. After incubation for 24 h at 37°C in 5% CO_2, the cells that had not penetrated the filter were completely wiped out with a cotton swabs, and the cells that had migrated to the lower surface of the filter were fixed with methanol. Then the cells were stained with H&E (Sigma), and counted in five randomly selected microscopic fields (×100) per filter.

Prepation of Conditioned Medium and Zymography. The equal number of cells was plated and maintained in 60-mm tissue culture plates with the media containing 10% FBS until subconfluency, and then the cells were washed three times with serum-free medium. The cells were then cultured for another 24 h in serum-free medium in the absence or presence of the various concentration of recombinant MIC-1. In certain experiments, various concentrations of PD98059 were cotreated with MIC-1. The conditioned media were collected after 24 h and clarified by centrifugation, and then concentrated by centrifugation through a Centricon Filter (10,000 molecular weight cutoff; Millipore, Beverly, MA). Gelatinolytic and fibrinolytic activities in cellular conditioned medium were analyzed by zymography as described previously (26, 27). Briefly, conditioned medium was mixed with SDS sample buffer without mercaptoethanol and heated for 30 min at 37°C. The amount of conditioned medium to be loaded on the zymography gel was normalized to cellular protein as assessed by a Bradford protein assay (Bio-Rad, Hercules, CA). Samples were electrophoresed in a 10% polyacrylamide gel containing 1 mg/ml of gelatin (for gelatin zymography) or gel containing 1 NIH unit/ml of thrombin, 12 mg/ml of fibrinogen, and 1 NIH unit/ml of plasminogen (for fibrin zymography). The gel was washed in 2.5% Triton X-100 to remove SDS, incubated at 37°C for 16 h in 200 mM NaCl containing 40 mM Tris-HCl and 10 mM $CaCl_2$ (pH 7.5), and stained with Coomassie Blue. The presence of gelatinolytic or fibrinolytic activity was identified as clear bands on a uniform blue background after destaining.

Northern Blotting. Total RNAs from the various cell lines were extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). Twenty μg of total RNA were electrophoresed in a 1% agarose-formaldehyde gels and transferred to nylon membranes (Roche, Mannheim, Germany). Specific probes for uPA, PAI-1, and uPAR were labeled with [α-^{32}P]dCTP by random priming method and hybridized with the membrane as described previously (25). After hybridization, the membrane was washed and exposed on film with an intensifying screen at −80°C.

Western Blot Analysis. Cells were washed with ice-cold PBS three times and extracted in ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 1% NP40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium vanadate, and 150 mM NaCl]. An aliquot of lysate was used to determine protein concentration by a Bradford protein assay. The equal amount of protein per lane was separated onto 10% SDS-polyacrylamide gel and transferred a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk for 3 h, and then incubated for 2 h with uPA antibody (Calbiochem), uPAR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a phosphospecific ERK1/2 antibody (BioLabs, Beverly, MA), or ERK1/2 antibody (BioLabs). After washing, the membrane was incubated with the secondary antibody conjugated with horseradish peroxidase. The signal was detected using the Amersham ECL system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Transfections and Luciferase Assay. 3TP-luciferase plasmid and control vector were transfected into SNU-620 cells using Lipofectamine plus reagent (Invitrogen Corporation). Twenty-four h after transfection, the transfected cells were treated with the indicated concentrations of TGF-β or MIC-1, and then incubated another 12 h. Luciferase activity was measured using luciferase assay system according to the instructions of the manufacturer (Promega, Madison, WI). Luciferase activity was determined in Microlumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 μl of assay buffer containing luciferin and measuring light emission for 10 s. The results were normalized to the activity of β-galactosidase expressed by cotransfected lacZ gene under the control of a constitutive promoter.

Proliferation Assay. The cells grown in RPMI 1640 supplemented with 10% FBS were placed in a 24-well plate at a concentration of 1×10^4/ml cells/well. After incubation for the indicated time, the viable cells were counted with a hemocytometer after trypan blue staining. In a certain experiment, the cells were cultured in the presence of TGF-β1 (1 ng/ml) or MIC-1 (20 ng/ml) for 72 h, and then the viable cells were counted. Results were calculated as the mean ± SD of triplicate cultures.

***In Situ* Hybridization.** Samples of gastric cancers (adenocarcinoma) were obtained from surgical cases in the Chungnam National University Hospital. The protocol used was approved by the Board of the Clinical Research Center at Chungnam National University Hospital, and informed written consent was obtained from all of the patients. Specimen from normal mucosa and invasive area of the cancer were fixed in 4% paraformaldehyde solution. *In situ* hybridization for *MIC-1* was performed with digoxigenin-labeled riboprobes. *MIC-1* antisense and sense riboprobes were synthesized from 926 bp human cDNA fragments cloned into pBluescript II KS vector (Stratagene) flanked by the T3 and T7 promoters. Frozen sections of each tissue were prepared and collected in PBS in a 24-well plate. The tissues were treated with proteinase K, acetylated with 0.25% acetic anhydride, prehybridized, hybridized with probe at a concentration of 0.5 μg/ml, treated with RNase A, and washed. The washed tissues were then incubated with antidigoxigenin alkaline phosphate-conjugated serum (diluted 1:500; Roche). The final coloring reaction was performed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution. Each sample section was mounted on a gelatin-coated slide and counterstained with 0.5% methyl green, dehydrated, and coverslipped with permount for viewing.

RESULTS

Overexpression of *MIC-1* in Gastric Cancer. To identify the secretary genes implicated in gastric cancer progression, we per-

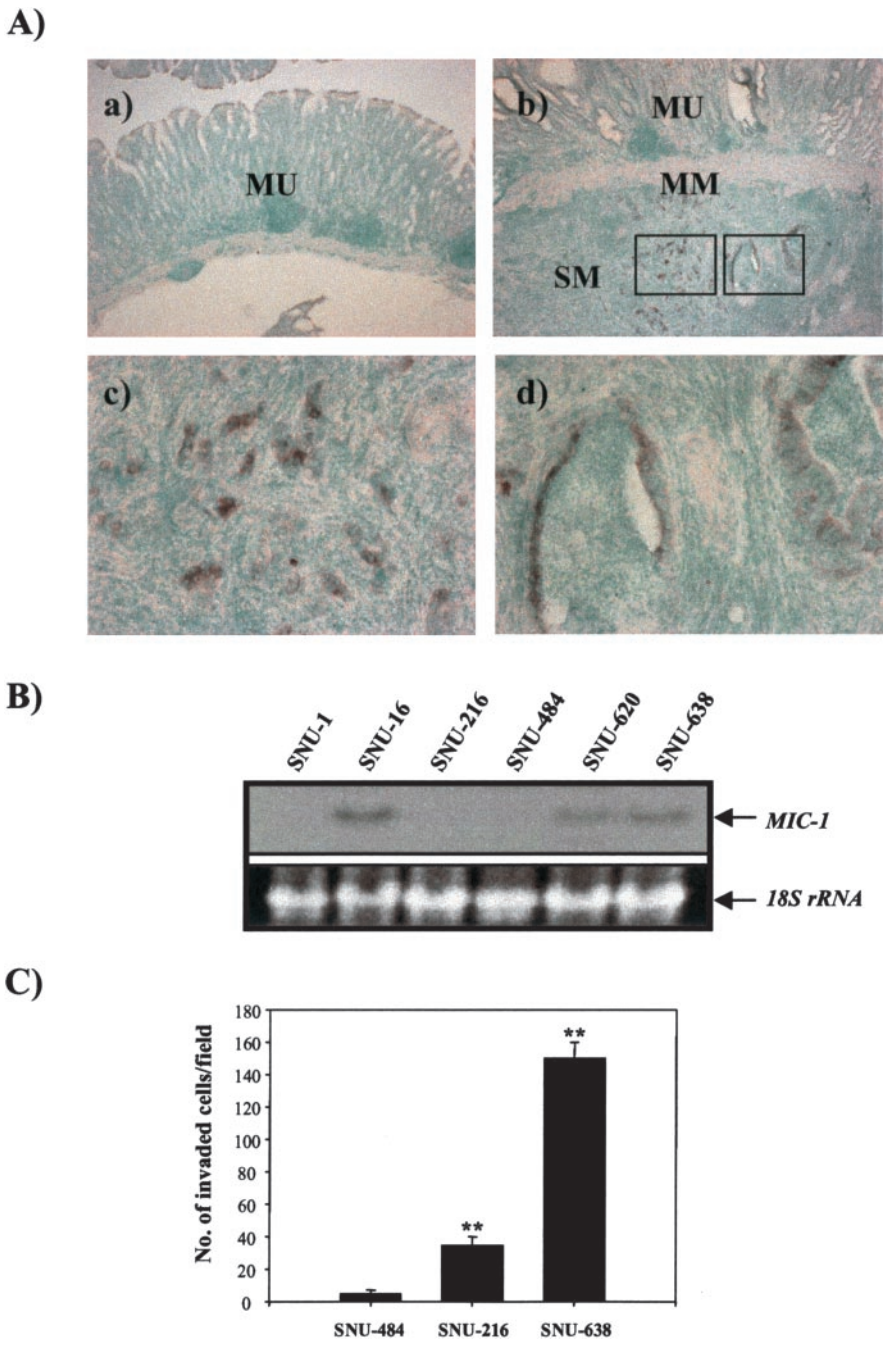

Fig. 1. Expression of MIC-1 in human gastric tumor tissues and cancer cell lines. *A*, analysis of MIC-1 mRNA expression in human gastric cancer (*MU*, mucosa; *MM*, muscularis mucosa; *SM*, submucosa). *a*, the mucosa of normal region (original magnification, ×40), *MIC-1* gene is not detected in the mucosa. *b*, the gastric tissues of invasive area (original magnification, ×40), *MIC-1* gene is detected in tumor cells in the submucosa. *c*, magnified photograph of the *left rectangle* in Fig. 1*A*, *panel b* (original magnification, ×200), *MIC-1* gene-positive cells form clusters consisting of a few tumor cells. *d*, magnified photograph of the *right rectangle* in Fig. 1*A*, *panel b* (original magnification, ×200), *MIC-1* gene-positive cells form glandular structure. *B*, analysis of MIC-1 mRNA expression in various human gastric cancer cell lines. To represent equal loading of nondegraded RNA, 18S rRNA stained with ethidium bromide was shown in the bottom. *C*, invasive potentials of human gastric cancer cell lines. Cells that migrate through the pores in the Matrigel-coated filter were fixed, stained, and counted in five random fields visualized by microscopy (×100). Data represent average of three independent experiments performed in triplicate; *bars*, ±SE; **, $P < 0.001$ *versus* SNU-484 cells (one-way ANOVA).

formed signal sequence trap using a cDNA library from the SNU-638 gastric cancer cell line and isolated 32 transcripts,[5] among which we identified *MIC-1* as a candidate gene. First, we analyzed the expression of *MIC-1* in 12 cases of surgically removed human gastric tumor tissues by *in situ* hybridization. Of these, 6 cases are poorly differentiated type, 4 cases are well-differentiated type, and 2 cases are the mixture of poorly and well-differentiated type. We found that *MIC-1* was specifically expressed in the tumor cells of all of the gastric cancer tissues examined, but not in their normal counterparts (Fig. 1). For example, *MIC-1* was not expressed in the normal mucosa of the specimen shown in Fig. 1*A*, *panel a*, and *MIC-1* was expressed in tumor cells in the submucosa of an invasive area in gastric cancer (Fig. 1*A*, *panel b*). *MIC-1*-positive tumor cells showed two expression patterns. MIC-1 transcript-positive cells form clusters consisting of a few tumor cells of a poorly differentiated type (Fig. 1*A*, *panel c*), or they form a glandular structure of a well-differentiated type (Fig. 1*A*, *panel d*). We also examined *MIC-1* expression by Northern blotting in human gastric cancer cell lines established from Korean cancer patients (23, 24). Most cancer cell lines derived from secondary tumor sites, including SNU-16, -620, and -638, expressed the MIC-1 transcript, but primary cancer cells SNU-1 and 484 did not (Fig. 1*B*). We examined whether expression level of MIC-1 was related with the invasiveness of gastric cancer cell lines, by investigating the invasive potentials of the cells using Matrigel-coated filters. We selected SNU-484, -216, and -638 cells, because SNU-1, -16, and -620 cells grow in suspension (23, 24), and did not adhere to Matrigel (data not shown). SNU-638 cells were more invasive than SNU-216 and -484 cells, which did not express *MIC-1* (Fig. 1*C*). These results led us to

[5] Unpublished observations.

hypothesize that the expression of *MIC-1* could affect the malignant progression of human gastric cancer cells.

Increased Invasiveness of SNU-216 Cells by the Ectopic Expression of MIC-1. To test this hypothesis, SNU-216 cells stably expressing *MIC-1* were established by retroviral infection with MIC-1 cDNA and designated SNU-216/MIC. To rule out the clonal variation of cells, we used pooled populations for additional experiments. The mRNA expression of exogenous *MIC-1* in SNU-216/MIC cells was confirmed by Northern blot analysis. The retrovirus-mediated 4.5-kb MIC-1 transcript was only detected in the SNU-216/MIC transfectants (Fig. 2*A*). We investigated whether the overexpression of MIC-1 could affect the invasiveness of SNU-216 cells, by investigating the ability of SNU-216/MIC to migrate through porous filters in response to a chemotactic stimulus, using a Transwell migration assay. SNU-216/MIC cells showed slightly increased migratory ability compared with parental SNU-216 and vector transfectants (Fig. 2*B*; $P < 0.05$, one-way ANOVA). However, in the invasion assay using Matrigel-coated filters, SNU-216 cells showed significantly increased invasiveness over the vector-transfected control ($P < 0.001$, one-way ANOVA). The number of invading cells was 5-fold higher for SNU-216/MIC than for the vector control (Fig. 2*C*; $P < 0.001$, one-way ANOVA). However, the proliferation of SNU-216/MIC cells was similar to that of parental SNU-216 and the vector transfectants (Fig. 2*D*). Next, we performed invasion assays using the conditioned medium from either SNU-216/MIC cells or control cells, and purified recombinant MIC-1. SNU-216 cells were treated for 24 h with the conditioned medium from SNU-216/MIC or from the control cells in the lower (Fig. 3*A*) or upper chambers (Fig. 3*B*) of a Boyden Transwell, and invasiveness was measured. SNU-216 cells only treated with the conditioned medium from SNU-216/MIC in the upper chamber showed significantly increased invasiveness. Moreover, recombinant MIC-1 significantly increased the invasiveness of SNU-216 cells in a dose-dependent manner, when it was treated in the upper chamber (Fig. 3*C*). Treatment of SNU-216 cells with 20 ng/ml of recombinant MIC-1 increased the invasiveness of the cells to almost a similar level to that of SNU-216/MIC cells. Taken together, these results suggest that MIC-1 could play a role as a mediator of gastric cancer cell invasion by stimulating their invasiveness.

MIC-1 Activates the Plasminogen Activator System of Gastric Cancer Cells. To address how MIC-1 stimulates gastric cancer cell invasiveness, we measured the proteolytic activities for SNU-216/MIC, and the control cells by gelatin and fibrin zymography (Fig. 4), and found that the SNU-216/MIC cells had higher gelatinolytic activity than the control cells (Fig. 4*A*). Interestingly, a dramatic increase of uPA activity was also observed in the SNU-216/MIC cells *versus* the control (Fig. 4*B*). Moreover, treatment of SNU-216 cells with recombinant MIC-1 significantly increased the uPA activity in a dose-dependent manner (Fig. 4*C*). To additionally examine the effect of MIC-1 on the uPA system, we analyzed the expressions of uPA, PAI-1, and uPAR by Northern and Western blot (Fig. 5*A*). SNU-216/MIC cells significantly increased the expressions of uPA and uPAR *versus* the control cells, but not that of PAI-1. Also, the level of an active form (33 kDa) of uPA was increased significantly in the conditioned medium of SNU-216/MIC-1 cells. To confirm these results, we investigated whether recombinant MIC-1 induced the expressions of uPA and uPAR (Fig. 5*B*). As expected, the treatment of SNU-216 cells with 20 ng/ml of MIC-1 significantly increased the expressions of uPA and uPAR in a time-dependent manner. Taken together, these results suggest that MIC-1 may stimulate the invasiveness of gastric cancer cells by up-regulating the uPA system.

MIC-1 Activates ERK1/2 Kinase. To investigate the mechanism of uPA activation by MIC-1, we decided to examine whether MIC-1 activates ERK1/2, as induced by several cytokines and growth factors (2). Accordingly, SNU-216 cells were treated with 20 ng/ml of recombinant MIC-1 for 5, 15, 30, 60, and 120 min, and the activation of ERK1/2 was then determined by Western blot (Fig. 6*A*). ERK1/2 was significantly activated in response to MIC-1. The level of the phosphorylated form of ERK1/2 reached a maximum at 5 min and returned to the basal level after 30 min. Furthermore, MIC-1 also

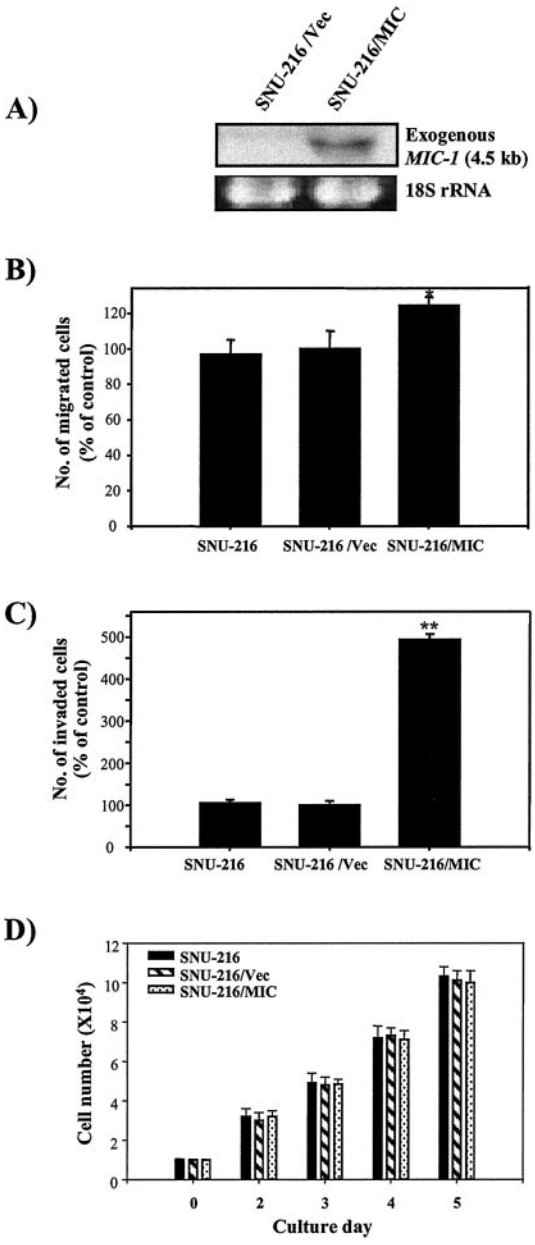

Fig. 2. Forced expression of MIC-1 into SNU-216 human gastric cancer cells stimulates its invasiveness. *A*, expression of MIC-1 mRNA by stably transfected SNU-216 cells as shown by Northern blotting. The SNU-216/Vec and SNU-216/MIC cell lines were stably transfected with either the empty vector or the MIC-1 expression vector, respectively. Twenty μg of total RNA was hybridized with a specific MIC-1 probe. The *bottom* represents 18S rRNA to show the equal loading of nondegraded RNA. *B* and *C*, effect of MIC-1 overexpression on cell migration and invasion of SNU-216 cells. Cells were plated on noncoated (*B*) or Matrigel-coated (*C*) filters for migration or invasion assay, respectively. Cells that migrate through the pores in the filter were fixed, stained, and counted in five random fields visualized by microscopy ($\times$100). Data represent average of three independent experiments performed in triplicate; *bars*, $\pm$SE; *, $P < 0.05$; **, $P < 0.001$ *versus* vector-transfectants (one-way ANOVA). *D*, effect of MIC-1 overexpression on proliferation of SNU-216 cells. The cells were plated at a concentration of 1×10^4/ml cells per well in a 24-well plate. After incubation for indicated periods of time, the viable cells were counted with a hemocytometer after trypan blue staining.

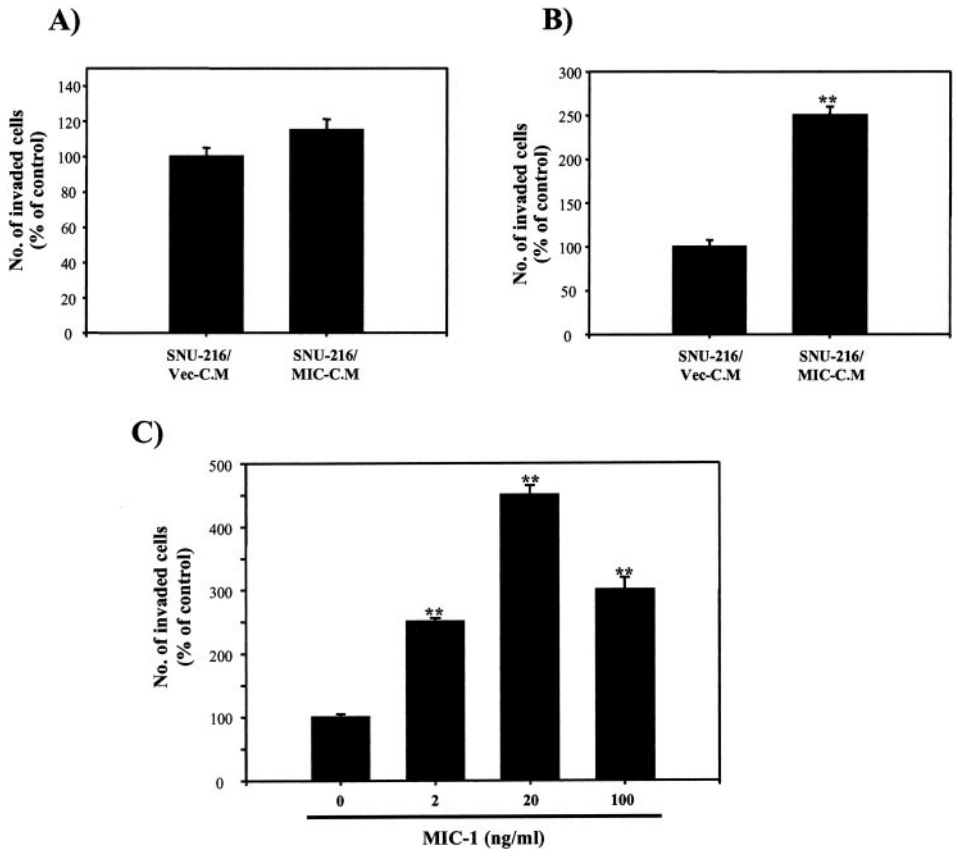

Fig. 3. Effect of MIC-1 on the invasiveness of SNU-216 cells. *A* and *B*, effect of conditioned medium on the invasiveness of SNU-216 cells when treated in the lower (*A*) or upper (*B*) chamber of Boyden Transwell. SNU-216/Vec-C.M, conditioned medium from SNU-216/Vec cells; SNU-216/MIC-C.M, conditioned medium from SNU-216/MIC cells. *C*, effect of recombinant MIC-1 on the invasiveness of SNU-216 cells. The cells were treated with various concentrations of purified recombinant MIC-1 in the upper chamber of Boyden Transwell. Cells that migrate through the pores in the Matrigel-coated filter were fixed, stained, and counted in five random fields visualized by microscopy (×100). Data represent average of three independent experiments performed in triplicate; *bars*, ±SE; **, $P < 0.001$ *versus* the control (one-way ANOVA).

activated MEK1/2, an upstream kinase of ERK1/2 (Fig. 6*B*). The level of the phosphorylated form of MEK1/2 reached a maximum at 2 min and returned to the basal level after 10 min. To investigate whether the activation of ERK1/2 in response to MIC-1 is critical for its effect on the invasiveness of gastric cancer cells, we examined the effects of PD98059, a specific MEK1/2 inhibitor, on the invasiveness of SNU-216 cells induced by MIC-1. Treatment of SNU-216 cells with PD98059 significantly inhibited the induced the activity of uPA, the amount of an active form of secreted uPA (33 kDa), and invasiveness of the cells by MIC-1 in a dose-dependent and consistent manner (Fig. 7, *A* and *B*). Moreover, PD98059 significantly inhibited the induced expression of uPA and uPAR by MIC-1 (data not shown). Taken together, these results suggest that MIC-1 may stimulate the invasiveness of gastric cancer cells by up-regulating the uPA system through, in part, the activation of ERK1/2.

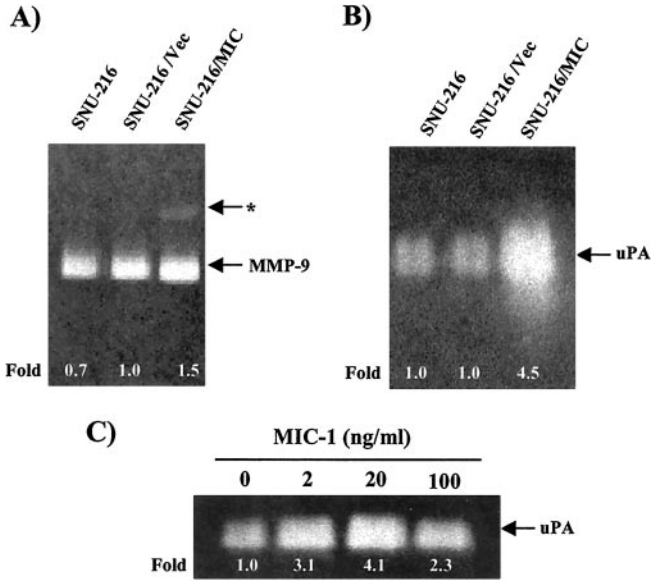

Fig. 4. Effect of MIC-1 on the gelatinolytic and fibrinolytic activities of SNU-216 cells. Conditioned medium from SNU-216, SNU-216/Vec, and SNU-216/MIC cells were performed gelatin (*A*) and fibrin zymography (*B*) as described in "Materials and Methods." *A*, an *arrow* indicates MMP-9 band, and an ∗ indicates unidentified protease band only appeared in the conditioned medium from SNU-216/MIC cells. *B*, an *arrow* indicates uPA band. *C*, SNU-216 cells were treated with the indicated concentrations of recombinant MIC-1, and then fibrin zymography was performed. An *arrow* indicates uPA band. *Fold* in the *bottom* of each zymogram represents the relative intensity of the band by densitometry.

MIC-1 Did Not Modulate the Proliferation of SNU-620, a TGF-β-responsive Gastric Cancer Cell Line. Because it has been reported previously that MIC-1 requires an intact signaling pathway mediated by TGF-β receptors to inhibit tumor cell growth (15), we investigated whether MIC-1 suppress gastric cancer cell growth using the well-characterized SNU-620 and SNU-638 gastric cancer cells. SNU-620 cells have functional TGF-β receptors and respond to TGF-β (28), whereas SNU-638 cells have mutated TGF- β receptors and do not respond to TGF-β (29, 30). As reported previously, we found that SNU-620 cells did respond to TGF-β, but that SNU-638 cells did not (Fig. 8*A*). Interestingly, recombinant MIC-1 did not suppress the proliferation of either cell line at the concentrations required to inducing invasiveness. Also, recombinant MIC-1 did not activate the TGF-β-responsive 3TP-luciferase plasmid, as assessed by a reporter assay (Fig. 8*B*). Moreover, recombinant MIC-1 was able to stimulate not only the activation of ERK1/2 (Fig. 8*C*), but also the uPA system and invasiveness in both SNU-638 and SNU-620 cell lines (data not shown). Taken together, these findings indicate that MIC-1 could exert its diverse biological effects through a signaling pathway other than TGF-β.

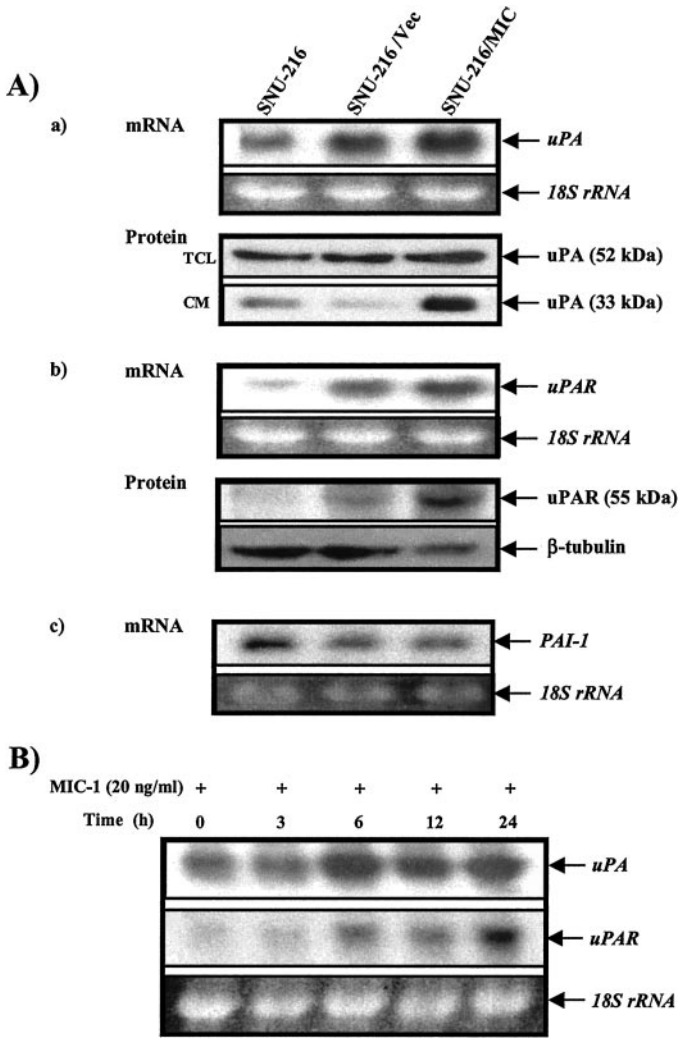

Fig. 5. Effect of MIC-1 on the expression of uPA, uPAR, and PAI-1 in SNU-216 cells. *A*, expressions of uPA, uPAR, and PAI-1 in SNU-216/MIC cells. *a*, expression of uPA; 18S rRNA was shown to represent the equal loading of nondegraded RNA; *TCL*, total cell lysates; *CM*, conditioned medium. *b*, expression of uPAR; to represent equal loading of protein or nondegraded RNA, tubulin or 18S rRNA stained with ethidium bromide was shown in *bottom* of each panel. *c*, expression of PAI-1 mRNA; the *bottom* represents 18S rRNA to show the equal loading of nondegraded RNA. *B*, effect of recombinant MIC-1 on the expression of uPA and uPAR in SNU-216 cells. The cells were treated with 20 ng/ml of recombinant MIC-1 for indicated periods of time, and then Northern blot analyses were performed. The *bottom* represents 18S rRNA to show the equal loading of nondegraded RNA.

DISCUSSION

Members of the TGF-β superfamily are multifunctional growth factors, and the nature of their effects depends on the cellular context (31). MIC-1 is a divergent member of the TGF-β superfamily and was first isolated from a subtracted cDNA library enriched with macrophage activation-associated genes (6, 7). The major function of the protein remains uncertain, although various biological activities of MIC-1 have been described (6, 9, 12, 15). In the present study, we provide evidence for the direct role of MIC-1 in gastric cancer invasion by activating the uPA system via the activation of ERK1/2. Furthermore, we found that is *MIC-1* specifically expressed in human gastric tumor cells and that the expression pattern of *MIC-1* is correlated with the invasive potential of various human gastric cancer cell lines. These findings have potential implications for MIC-1 with respect to its role in the invasive and metastatic progression of human gastric cancer cells.

The acquisition of tumor cell invasiveness is an important aspect of tumor progression, and a principal factor of cancer morbidity and mortality. In studies conducted in a number of experimental models, it appears that cell migration and the production of proteases, including uPA and MMPs, are essential components of the invasion process.

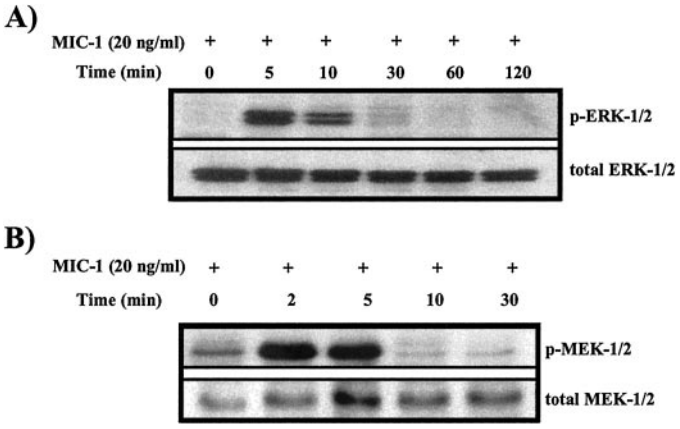

Fig. 6. Effect of MIC-1 on MEK-ERK activation. SNU-216 cells were treated with 20 ng/ml of recombinant MIC-1 for indicated periods of time. Cells were lysed, and the levels of phosphorylated ERK-1/2 (p-ERK-1/2, *A*) and MEK-1/2 (p-MEK-1/2, *B*) were determined by Western blot analysis with corresponding phospho-specific antibodies. The *bottom* represents total ERK-1/2 (*A*) and MEK-1/2 (*B*) to show the equal loading of cell lysates as determined by Western blot analysis.

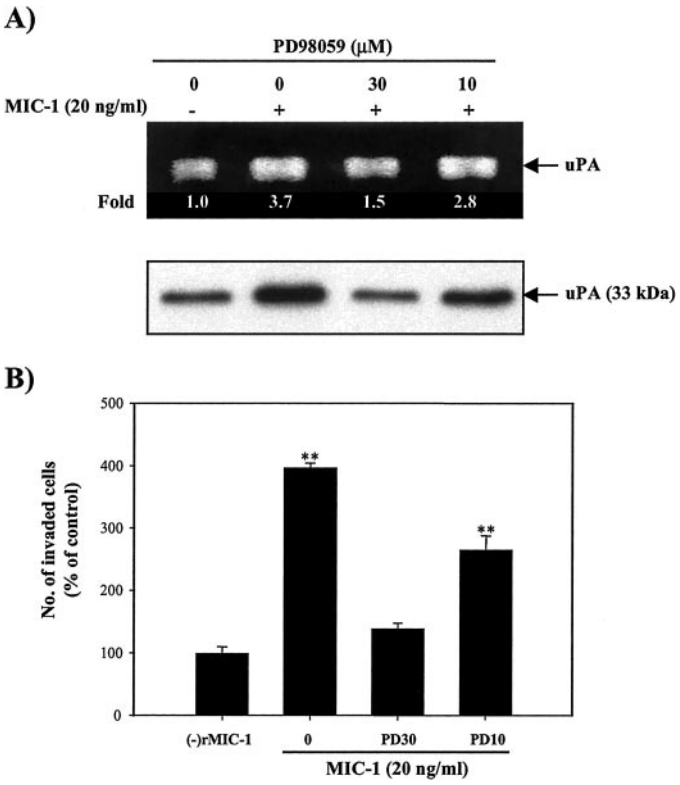

Fig. 7. PD98059, a specific MEK-1/2 inhibitor, blocks the induced uPA activity and invasion of SNU-216 cells by MIC-1. *A*, effect of PD98059 on the uPA activity induced by MIC-1. Cells were treated with 20 ng/ml of recombinant MIC-1 in the presence or absence of indicated concentrations of PD98059 and the uPA activity (*top*) measured as described in "Materials and Methods," and the amount of secreted active form of uPA (*bottom*) from each sample was determined by Western blot. *Fold* in the *bottom* of the zymogram represents the relative intensity of the band by densitometry. *B*, effect of PD98059 on the invasiveness induced by MIC-1. Cells were plated on Matrigel-coated filters in combinations with recombinant MIC-1 and PD98059 as indicated. *PD10*, 10 μM of PD98059; *PD30*, 30 μM of PD98059. Cells that migrate through the pores in the filter were fixed, stained, and counted in five random fields visualized by microscopy ($\times$100). Data represent average of three independent experiments performed in triplicate; *bars*, $\pm$SE; **, $P < 0.001$ *versus* the control (one-way ANOVA).

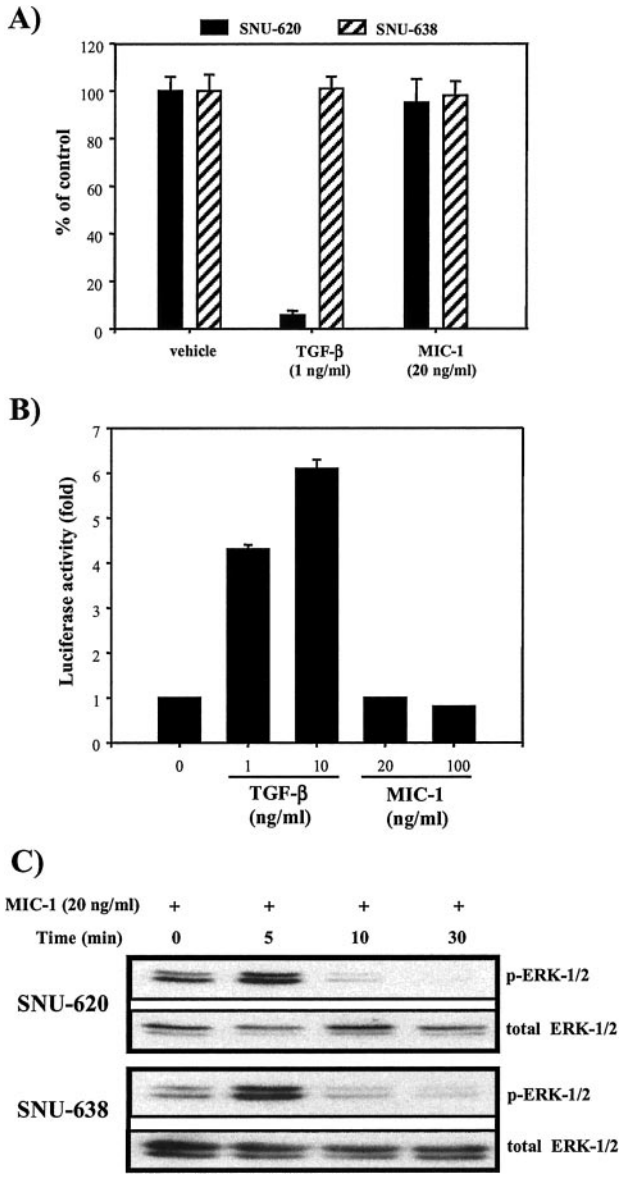

Fig. 8. Effect of MIC-1 on TGF-β-responsive and -unresponsive gastric cancer cell lines. *A*, recombinant MIC-1 does not modulate the proliferation of TGF-β-responsive SNU-620 and TGF-β-unresponsive SNU-638 cells. The cells were treated with indicated concentrations of TGF-β or MIC-1 for 72 h, and then the viable cells were determined as described in "Materials and Methods." *B*, recombinant MIC-1 does not activate TGF-β-responsive 3TP-luciferase plasmid. SNU-620 cells were transiently transfected with 3TP-luciferase plasmid and then stimulated the indicated concentrations of TGF-β or MIC-1. The luciferase activities were determined as described in "Materials and Methods." *C*, recombinant MIC-1 activates ERK-1/2 in both SNU-620 and SNU-638 cells. The cells were treated with 20 ng/ml of purified recombinant MIC-1 for indicated periods of time, and then the level of phosphorylated ERK-1/2 (*p-ERK-1/2*) were determined by Western blot analysis with a phospho-specific antibody. The *bottom* represents total ERK-1/2 to show the equal loading of cell lysates as determined by Western blot analysis; *bars*, $\pm$SE.

Transfection of *MIC-1* into human gastric cancer SNU-216 cells slightly increased migratory potential of the cells, but strongly increased the invasive potential of the cells. Consistently, a dramatic increase of invasiveness of SNU-216 cells was observed only when the conditioned medium from SNU-216/MIC cells or recombinant MIC-1 was added to the upper chamber of transwell. These results imply that MIC-1 could induce the invasiveness of the cells by directly stimulating invasive machinery such as proteases rather than migration, and it could not work as a chemoattractant for human gastric cancer cells. Among the many proteases involved in invasion, uPA and uPAR are of particular importance (2, 3). uPA is a serine protease and, when bound to its receptor, initiates the activation of MMPs as well as the conversion of plasminogen to plasmin. These proteases confer on the cells the ability to degrade the ECM, thus allowing them to overcome the constraints of cell-cell and cell-matrix interactions. uPA and uPAR are known to be overexpressed in various malignancies including breast, ovarian, and gastric cancers, and have been demonstrated to be essential in the maintenance of invasive and metastatic phenotypes (3, 5). Furthermore, a good correlation has been observed between the expression of the uPA system and malignant progression in a variety of cancers, including gastric cancer (32), although the mechanism involved in the maintenance of uPA and uPAR expression in cancer cells remains largely unknown. The present study demonstrates that the transfection of *MIC-1* into human gastric cancer SNU-216 cells stimulates the expression and activity of the uPA system, and the invasion of gastric cancer cells *in vitro*. Consistent with these results, recombinant MIC-1 also exhibited the similar effects to those of *MIC-1* transfection. Therefore, it is likely that MIC-1 could act in an autocrine/paracrine manner to maintain the invasive phenotype of gastric cancer cells by stimulating the uPA system.

We also found that *MIC-1* is specifically expressed in gastric tumor tissues. Although the significance of *MIC-1* expression in tumor cells remains to be elucidated, *MIC-1* has been identified as an overexpressed gene in tumor tissues in prostate (20) and colorectal cancers (22), and also as an up-regulated gene during the progression of androgen-independent growth in human prostate cancer cells (21). Also, as reported in glioblastoma cells (33), our preliminary results showed that *MIC-1* was significantly up-regulated in SNU-216 cells by hypoxia (data not shown), which plays an important role in malignant progression of a variety of cancers (34). Taken with the results of our present study, it is likely that the expression of MIC-1 in tumor cells functions as a promoter of tumor progression by inducing the cancer cell invasiveness.

In an effort to investigate the molecular mechanisms underlying the simultaneous expression of uPA system by MIC-1 expression, we were able to demonstrate that ERK1/2 is strongly activated in response to MIC-1. Furthermore, treatment with PD98059, a specific MEK inhibitor, nearly abolished the invasiveness of gastric cancer cells and their uPA activity, as well as the mRNA expression of uPA and uPAR. In line with our results, it has been reported that the mRNA expression of uPA and uPAR can be up-regulated by mitogen, growth factors, and oncogenes through a signaling pathway that activates ERK1/2 (2). Our results provide additional evidence that the ERK1/2 signaling pathway may play an important role in the regulation of uPA and uPAR expression in cancer cells.

Recent reports revealed that transfection of MIC-1 cDNA into human colorectal carcinoma HCT-116 cells or into a human glioblastoma LN-Z308 cell line suppressed tumor growth *in vitro* or *in vivo*, although the authors did not provide evidence that recombinant MIC-1 exerts antitumor activity in their system (11, 33). Also, it has been reported that MIC-1, like TGF-β, requires an intact signaling pathway mediated by TGF-β receptors, as well as receptor-activated Smad4 to suppress tumor cell growth, although very high concentrations of MIC-1protein (IC_{50} = 0.8 μg/ml) were required to elicit this effect (15). In the present study, we were unable to demonstrate that MIC-1 suppresses gastric cancer cell growth in gastric cancer cell lines, regardless of TGF-β responsiveness. However, we have shown that MIC-1 can induce the invasion of gastric cancer cells by activating the uPA system. Furthermore, recombinant MIC-1 induced cancer cell invasion and the activation of the uPA system, without significantly affecting the cell proliferation, in a dose-dependent manner. Therefore, it is likely that MIC-1, like TGF-β (35), could function

both positively and negatively on tumorigenesis, and that this depends on the molecular and cellular contexts of the cells. Importantly, it is worth noting that MIC-1 induced the activation of ERK1/2 and of the uPA system independent of TGF-β receptors. Moreover, MIC-1 did not activate the TGF-β-responsive 3TP-luciferase plasmid, which has been shown to be induced by various members of the TGF-β superfamily (36, 37). Also, pretreatment of SNU-620 cells with recombinant MIC-1 did not significantly modulate the inhibition of the TGF-β-mediated proliferation of SNU-620 cells (data not shown). This raises the possibility that MIC-1 acts through the different signaling pathway from TGF-β. Therefore, we feel that a detailed analysis of MIC-1 signaling pathway in gastric cancer cells could lead to an enhanced understanding of the potential role played by MIC-1 in cancer progression. We are now additionally investigating this signaling pathway.

Taken together, our results may have functional implications in terms of gastric cancer cell invasion. Any potential up-regulation of uPA and uPAR by MIC-1 in gastric cancer cells is likely to promote the ability of tumor cells to invade their surrounding environment, because uPA ligation to uPAR may trigger proteolytic pathways and, thus, the ability of tumor cells to degrade the ECM. Our findings that MIC-1 regulates the levels of expressions of uPA and uPAR open a new avenue for investigating the close linking between MIC-1 signaling, the uPA system and gastric cancer cell invasion, and possibly metastasis.

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