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Macrophage inhibitory cytokine-1 transactivates ErbB family receptors via the activation of Src in SK-BR-3 human breast cancer cells

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The function of macrophage inhibitory cytokine-1 (MIC-1) in cancer remains controversial, and its signaling pathways remain poorly understood. In this study, we demonstrate that MIC-1 induces the transactivation of EGFR, ErbB2, and ErbB3 through the activation of c-Src in SK-BR-3 breast cells. MIC-1 induced significant phosphorylation of EGFR at Tyr845, ErbB2 at Tyr877, and ErbB3 at Tyr1289 as well as Akt and p38, Erk1/2, and JNK mitogen-activated protein kinases (MAPKs). Treatment of SK-BR-3 cells with MIC-1 increased the phosphorylation level of Src at Tyr416, and induced invasiveness of those cells. Inhibition of c-Src activity resulted in the complete abolition of MIC-1-induced phosphorylation of the EGFR, ErbB2, and ErbB3, as well as invasiveness and matrix metalloproteinase (MMP)-9 expression in SK-BR-3 cells. Collectively, these results show that MIC-1 may participate in the malignant progression of certain cancer cells through the activation of c-Src, which in turn may transactivate ErbB-family receptors. [BMB reports 2010; 43(2): 91-96]

INTRODUCTION

MIC-1, which is identical to placental transforming growth factor-β (PTGF-β), placental bone morphogenic protein (PLAB), growth differentiation factor-15 (GDF-15), prostate-derived factor (PDF), or nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1), is a divergent member of the TGF-β superfamily (1, 2). MIC-1 is expressed quite widely; but under resting conditions, the placenta is the only tissue that expresses large quantities of MIC-1 (2). The epithelial cells express lower quantities of MIC-1 mRNA. MIC-1 expression is, however, dramatically increased in cases of inflammation, injury, and malignancy (3). The principal function, receptor, and signaling pathway of MIC-1 remain uncertain, although several of its biological activities have already been described (2, 4-6). The role of MIC-1 in cancer also remains poorly understood. Increased MIC-1 expression is a common feature of many cancers. Several studies have observed major upregulation of MIC-1 mRNA and protein in cancer biopsies (7-9). Serum MIC-1 levels are often markedly elevated in cases of metastatic cancer, and appear to occur in parallel with the stages and extent of disease, particularly in cases of colorectal cancer (9-11). Paradoxically, a number of studies have described an antitumorigenic function for MIC-1, by which it induces apoptosis and may negatively affect tumor growth (12-15). However, our lab and others have shown that MIC-1 promotes development of more aggressive cancers (16-18). MIC-1 can induce invasiveness of human gastric cancer cells via the activation of Akt and ERK1/2 (16, 17), and increase the tumorigenicity of melanoma cells (18). It has also been reported that MIC-1 mediates tumor-induced anorexia and weight loss in prostate cancer (19).

The family of ErbB receptor tyrosine kinases includes four members: epidermal growth factor receptor (EGFR)/ErbB1, ErbB2/Neu/HER2, ErbB3, and ErbB4. The binding of peptides of the EGF-related growth factor family to the extracellular domain of the ErbB receptors causes the formation of homo- and heterodimers. Ligand binding induces intrinsic receptor kinase activity, ultimately resulting in the stimulation of intracellular signaling cascades, including the phosphatidylinositol-3 kinase (PI3K)/Akt and MAPKs cascade (20, 21). In addition, ErbB family receptors can be transactivated by various extracellular stimuli, such as agonists for G protein-coupled receptors (GPCR) and cytokine receptors (22, 23). For example, CXCL-12 and interleukin-6 have been reported to transactivate EGFR and ErbB2 in a variety of cancer cell types (24, 25). Members of the Src family of nonreceptor intracellular tyrosine kinases have been implicated in ErbB receptor transactivation via direct phosphorylation of cytoplasmic domains of EGFR or via stimulation of matrix metalloproteinase (MMP) activity to promote the release of the membrane-bound EGFR ligand (26-30).

As described herein, we report that MIC-1 induced the transactivation of EGFR, ErbB2, and ErbB3 receptor tyrosine kinases

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in SK-BR-3 human breast cancer cells. This activation was dependent on Src activation. These novel observations provide additional support for the notion that MIC-1 may operate as a positive regulator of tumor progression.

RESULTS AND DISCUSSION

MIC-1 activates EGFR, ErbB2, and ErbB3 in SK-BR-3 cells

Not a great deal is currently known about the MIC-1-induced signaling pathways, and the receptor for MIC-1 remains to be identified. In order to further investigate the MIC-1 signaling pathway, we assessed the effects of MIC-1 on EGFR, ErbB2, and ErbB3 phosphorylation using phospho-specific antibodies in SK-BR-3 cells (Fig. 1A). MIC-1 significantly induced phosphorylation of EGFR at Tyr845, ErbB2 at Tyr877, and ErbB3 at Tyr1289 in a time-dependent manner. MIC-1 stimulation increased the levels of phosphorylation of EGFR, ErbB2, and ErbB3 with the peak at 2 min, 10 min, and 5 min, respectively. Dose-response experiments revealed that MIC-1 increased the phosphorylation levels of EGFR, ErbB2, and ErbB3, with maximum induction at a dose of 20 ng/ml (Fig. 1B). Since it is well known that activation of EGFR family receptors results in the activation of a variety of signaling cascades, including the PI3K/Akt and MAPKs cascade (20, 21), we determined whether MIC-1 could activate Akt and p38, JNK, and Erk1/2 MAPKs (Fig. 1C). MIC-1 increased the phosphorylation levels of Akt and p38, JNK, and Erk1/2 MAPKs. These results indicate that MIC-1 may induce EGFR, ErbB2, and ErbB3 activation, and thereby activates Akt and MAPKs signaling pathways.

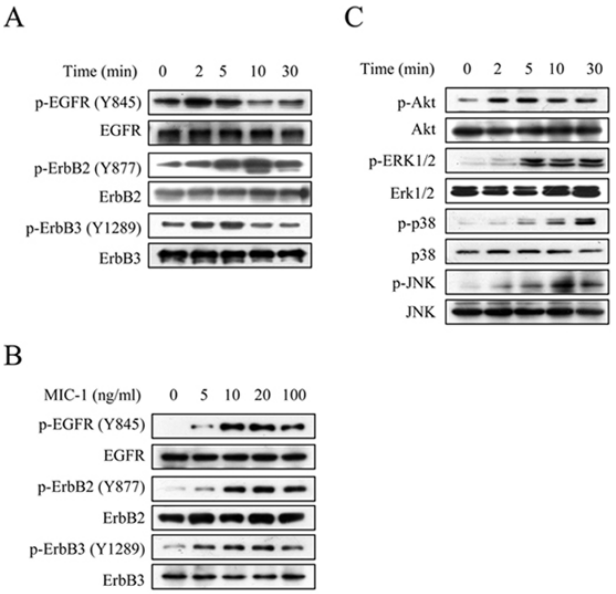

Fig. 1. MIC-1 activates ErbB family receptors. (A, B) SK-BR-3 cells were serum-starved for 12 h, followed by stimulation with 20 ng/ml of MIC-1 for indicated periods of time (A), or indicated concentrations of MIC-1 for 5 min (B). The levels of phospho-EGFR, EGFR, phospho-ErbB2, ErbB2, phospho-ErbB3, and EbB3 were determined by Western blot analysis. (C) SK-BR-3 cells were serum-starved for 12 h, followed by stimulation with 20 ng/ml of MIC-1 for indicated periods of time. Whole cell lysates were subjected to Western blot analysis with phospho-Akt, phospho-Erk1/2, phospho-p38, or phospho-JNK antibodies. The same membranes were stripped and reblotted with Akt, Erk1/2, p38, or JNK antibody.

Src activity is required for MIC-1-induced EGFR, ErbB2, and ErbB3 activation

It is known that one mechanism of transactivation of ErbB family receptors includes activation of Src-family protein kinases (26-30). Activated Src is able to activate EGFR directly through the phosphorylation of EGFR at Tyr845 (27, 30), and enhances

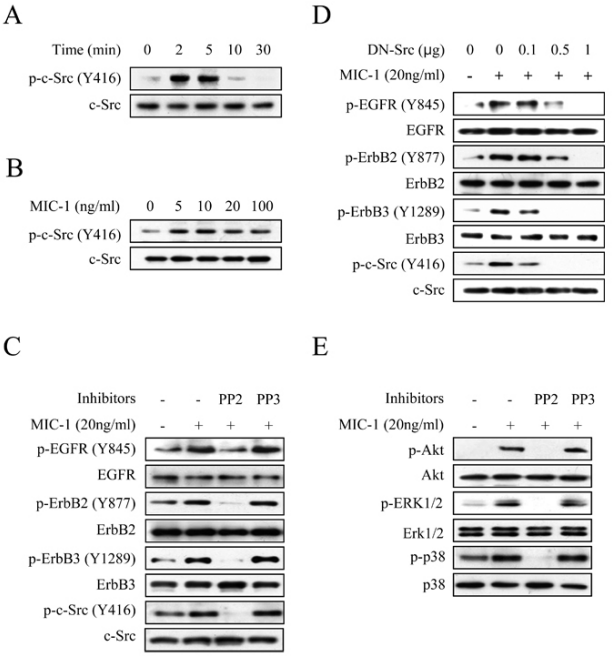

Fig. 2. MIC-1-induced Src activity is necessary for activation of ErbB-family receptors. (A, B) Serum-starved SK-BR-3 cells were stimulated with 20 ng/ml of MIC-1 for indicated periods of time (A), or indicated concentrations of MIC-1 for 5 min (B). Levels of phospho-Src and Src were determined by Western blot analysis. (C) Serum-starved SK-BR-3 cells were stimulated with 20 ng/ml of MIC-1 in the presence of vehicle, PP2 (10 μM), or PP3 (10 μM) for 5 min. The levels of phospho-EGFR, EGFR, phospho-ErbB2, ErbB2, phospho-ErbB3, EbB3, phospho-Src, and Src were determined by Western blot analysis. (D) SK-BR-3 cells transfected with an indicated amount of a dominant negative Src expression vector (DN-Src) were serum-starved for 12 h, and followed by stimulation with 20 ng/ml of MIC-1 for 5 min. The levels of phospho-EGFR, EGFR, phospho-ErbB2, ErbB2, phospho-ErbB3, EbB3, phospho-Src, and Src were determined by Western blot analysis. (E) Whole-cell lysates prepared in (C) were subjected to Western blot analysis to determine the levels of phospho-Akt, Akt, phospho-Erk1/2, Erk1/2, phospho-p38, and p38.

ErbB2/ErbB3 signaling by promoting ErbB2/ErbB3 heterocomplex formation (28). Therefore, we examined whether MIC-1 may activate Src (Fig. 2A). The phosphorylation level of Src at Tyr416 was significantly increased by MIC-1 treatment. Furthermore, this activation was dose-dependent (Fig. 2B). Next, we determined whether inhibition of Src activity attenuates MIC-1-induced phosphorylation of EGFR, ErbB2, and ErbB3 (Fig. 2C). Treatment of the cells with PP2, a specific pharmacological inhibitor of Src, significantly inhibited the phosphorylation of EGFR, ERbB2, and ErbB3, but PP3, an inactive derivative of PP2, did not. Likewise, transfection with a dominant negative of Src also suppressed MIC-1-induced phosphorylation of EGFR, ErbB2, and ErbB3 (Fig. 2D). Inhibition of Src activity by PP2 also decreased MIC-1-induced activation of Akt and p38, and Erk1/2 MAPKs (Fig. 2E). These results suggest that MIC-1 may induce transactivation of EGFR, ErbB2, and ErbB3 via the activation of Src tyrosine kinase.

Inhibition of Src activity suppresses MIC-1-induced invasiveness of SK-Br-3 cells

In order to confirm that Src activity is required for MIC-1-induced activation of signaling pathways, we measured whether inhibition of Src activity modulates MIC-1-induced invasiveness of SK-BR-3 cells (Fig. 3A). Pharmacological inhibition of Src activity by PP2 resulted in a significant abrogation of MIC-1-induced invasiveness of the cells, as AG825, an ErbB2 inhibitor, did (17). Moreover, zymography and immunoblot analysis also revealed that MIC-1, but not MMP-2, significantly induced MMP-9 expression, and that this induction was completely suppressed by treatment with PP2 (Fig. 3B).

MIC-1 is a divergent member of the TGF-β superfamily, and its principal function remains to be precisely determined. Although there is a strong correlation between MIC-1 expression and epithelial tumors, less is currently known regarding its role and the manner in which it exerts its effects. Significant increases have been noted in serum levels of MIC-1 with tumor progression to metastatic disease in several epithelial cancers, including colon, prostate, and pancreatic cancer (3). We previously reported that elevated MIC-1 expression in gastric cancer cell lines was associated with a more invasive phenotype (16), and that MIC-1 induced the expression of the hypoxia inducible factor-1α protein and the expression of its target genes, via the activation of the mammalian target of rapamycin signaling pathway (17).

In this study, we have uncovered evidence for a role of MIC-1 that underlies the induction of tumor progression on the basis of the activation of ErbB family receptors and Src. MIC-1 profoundly induces the transactivation of EGFR, ErbB2, and ErbB3 receptor tyrosine kinses, and this activation is inhibited effectively by the inhibition of Src activity in SK-BR-3 cells. Moreover, we showed that MIC-1 induces invasiveness of SK-BR-3 cells, and inhibition of Src activity results in significant suppression of MIC-1-induced invasiveness as well as MMP-9 activation. Collectively, these data provide evidence

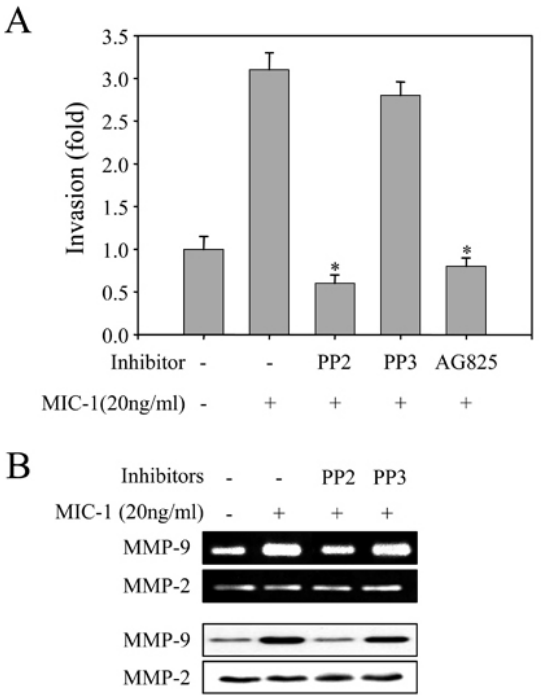

Fig. 3. Inhibition of Src activity suppresses MIC-1-induced invasiveness of SK-BR-3 cells. (A) SK-BR-3 cells were plated in the upper chamber of culture well inserts, and then treated with the 20 ng/ml of MIC-1 in the presence of vehicle, PP2 (10 μM), PP3 (10 μM), or AG825 (50 μM) for 24 h. Cells that migrate through the pores in the 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, SD of triplicate samples from three independent experiments; *statistically significant ($P < 0.005$, Student's t test) versus control. (B) The conditioned media were prepared form SK-BR-3 cells treated with 20 ng/ml of MIC-1 in the presence of vehicle, PP2 (10 μM), or PP3 (10 μM) for 24 h. The activities for MMP-9 and MMP-2 were measured by gelatin zymography (upper panel), and the amounts of secreted MMP-9 and MMP-2 were determined by Western blot analysis (lower panel).

that the signaling pathways of MIC-1 operate as a mediator of tumor progression in human breast cancer cells.

How might MIC-1 activate EGFR, ErbB2, and ErbB3 receptor tyrosine kinase? Thus far, the receptor for MIC-1 has yet to be identified. MIC-1 may not be a ligand for EGFR or ErbB3 (13). ErbB family receptors have been shown to be transactivated by several factors (24, 25). For example, CXCL-12, the receptor of which is a member of a family of G-protein-coupled receptors, transactivates EGFR and ErbB2 in MBA-MB-361 and SK-BR-3 human breast cancer cells via Src kinase activation (25). Src is able to phosphorylate EGFR at Tyr845 and ErbB2 at Tyr877 in the activation loop of the kinase domain, and this phosphorylation enhances EGFR and ErbB2 kinase activity (29, 30). Src activity also enhances heterocomplex formation of ErbB2 and ErbB3 that results in increased basal and/or ligand-induced ac-

tivation of receptors, and their downstream intracellular effectors (28).

In the present study, we showed that MIC-1 induced phosphorylation of EGFR at Tyr845, ErbB2 at Tyr877, and ErbB3 at Tyr1289 dependent on Src tyrosine kinase activity. Therefore, it can be speculated that MIC-1 may transactivate ErbB family receptor tyrosine kinases via Src activation after binding to its own receptor. The mechanism underlying this activation should be described in detail, but it cannot until the MIC-1 receptor has been identified.

MMP-9 plays a key role in physiological processes such as development, wound healing, angiogenesis, and also in pathological processes such as inflammation, tumor invasion, and metastasis (31). Elevated expression of MMP-9 is associated with increased metastatic potential in many cancer types, including breast cancers (32). Several studies have demonstrated that activation of ErbB family receptors induces MMP-9 expression and invasiveness of human breast cancer cells (33, 34). Our results showed that MIC-1 can induce MMP-9 expression through Src-dependent activation of ErbB-family receptors in SK-BR-3 cells. This MIC-1-induced MMP-9 expression may allow breast cancer cells to increase invasive potential, thereby enhancing their tumor progression ability.

In conclusion, our results indicate that MIC-1 may positively affect tumor progression via the Src-dependent transactivation of ErbB family receptors. Any activation of ErbB family tyrosine kinases by MIC-1 is likely to promote the ability of tumor cells to activate oncogenic signaling, most notably signaling of Akt and MAPKs.

MATERIALS AND METHODS

Cell culture

Human breast cancer SK-BR-3 cells were purchased from American Type Culture Collection and maintained in RPMI 1640 supplemented with penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Reagents

Human recombinant MIC-1 was prepared as previously described (16). A dominant negative Src expression vector was kindly provided by Prof. H. Lee (Kangwon National University). Specific antibodies to phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-p38 (Thr180/Tyr182), p38, phospho-JNK (Thr183/Tyr185), JNK, phospho-Akt (Ser473), Akt, phospho-EGFR (Tyr845), phospho-ErbB2 (Tyr877), phospho-ErbB3 (Tyr 1289), phospho-Src (Tyr416), and Src were purchased from Cell Signaling Technology (Danvers, MA). EGFR (1005), ErbB2 (C-18), ErbB3 (C-17), and MMP-9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). AG825, PP2, and PP3 were purchased from Calbiochem (San Diego, CA).

Western blot analysis

Cells were lysed with a buffer [50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 2 mM benzamidine, 50 mM NaF, 5 mM sodium orthovanadate, and 150 mM NaCl]. Proteins were separated by SDS-PAGE and transferred a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% skim milk at room temperature for 2 h, and then incubated for 2 h with primary antibodies. After washing, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. The signal was detected using the enhanced chemiluminescence system (Intron, Seongnam, Korea).

Invasion assays

The ability of cells to invade through Matrigel-coated filters was determined using a modified 24-well Boyden chamber (Corning Costar, Cambridge, MA; 8 μm pore size) as previously described (16, 17). Briefly, SK-BR-3 cells were seeded at a density of 5×10^4 cells in 100 μl RPMI 1640 containing 10% FBS in the upper compartment of a transwell, and then exposed to 20 ng/ml of MIC-1 for 24 h. 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 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 media. The cells were then treated with MIC-1 for another 24 h in the absence or presence of various inhibitors. The conditioned media were collected and clarified by centrifugation, and then concentrated by centrifugation through a Centricon Filter (10,000 molecular weight cutoff, Millipore, Beverly, MA). Gelatinolytic activities in cellular conditioned media were analyzed by zymography as previously described (16, 35). Briefly, the conditioned medium was mixed with SDS sample buffer without mercaptoethanol and incubated for 30 min at 37°C. Samples were electrophoresed in a 10% polyacrylamide gel containing 1 mg/ml of gelatin. 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 activity was identified as clear bands on a uniform blue background following destaining.

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