Regulation of hepatocyte growth factor-mediated urokinase plasminogen activator secretion by MEK/ERK activation in human stomach cancer cell lines

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Abbreviations: ERK, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; MAPK, mitogen activated protein kinase; MEK1, MAPK/ERK kinase 1; uPA, urokinase plasminogen activator

Abstract

The regulatory mechanisms for the proliferation and the particular invasive phenotypes of stomach cancers are not still fully understood. Up-regulations of hepatocytes growth factor (HGF), its receptor (c-Met), and urokinase-type plasminogen activator (uPA) are correlated with the development and metastasis of cancers. In order to investigate roles of HGF/c-Met signaling in tumor progression and metastasis in stomach cancers, we determined effects of a specific MEK1 inhibitor (PD098059) and a p38 kinase inhibitor (SB203580) on HGF-mediated cell proliferation and uPA expression in stomach cancer cell lines (NUGC-3 and MKN-28). HGF treatment induced the phosphorylations of ERK and p38 kinase in time- and dose- dependent manners. Pre-treatment with PD098059 reduced HGF-mediated cell proliferation and uPA secretion. In contrast, SB203580 pre-treatment enhanced cell proliferation and uPA secretion due to induction of ERK phosphorylation. Stable expression of dominant negative-MEK1 in NUGC-3 cells showed a decrease in

HGF-mediated uPA secretion. These results suggest that interaction of a MEK/ERK and a p38 kinase might play an important role in proliferation and invasiveness of stomach cancer cells.

Keywords: hepatocyte growth factor; mitogen-activated protein kinases; neoplasm metastasis; urinary plasminogen activator

Introduction

Gastric cancer still remains the leading cause of cancer death in Korea because of the rapidly invasive and metastatic progression of the cancer cells. However, the mechanism for the procession of gastric cancer is still poorly understood. The over-expression of uPA has been detected in various malignancies including breast (Miyake *et al.*, 1999), prostate (Yang *et al.*, 2000), colon (Duggan *et al.*, 1995), and pancreatic (Lee *et al.*, 2003b). Some data have shown that a high level of uPA in tumors is associated with a rapid disease progression and a poor prognosis (Solomayer *et al.*, 1997; Bouchetm *et al.*, 1998). In addition, studies performed with both *in vitro* and *in vivo* experimental models have demonstrates that the levels of uPA are closely associated with the degree of tumor cell invasion (Xing *et al.*, 1996). Blocking uPA expression or disruption of uPA binding to uPAR has been found to significantly inhibit tumor cell invasion and metastasis in various tumor models (Alonso *et al.*, 1996; Krieger *et al.*, 2000). From these facts, it is apparent that uPA plays a key role in tumor progression and metastasis. We recently demonstrated a cellular mechanism for invasion-metastasis via hepatocyte growth factor/c-Met signaling in human gastric cells together with the induction of the urokinase proteolysis network.

The mitogen-activated protein kinases (MAPK) transduce extracellular signals into cellular responses, and so they play important roles in proliferation, apoptosis, differentiation, migration and cytoskeleton remodeling of cells (English *et al.*, 1999; Widmann *et al.*, 1999).

There are at least three distinct MAP kinase signal transduction pathways in mammalian cells that lead to activation of either the extracellular regulated kinases (ERKs); the c-Jun and N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway

or the p38 MAPK pathway. Activated ERK subsequently transmits the mitogenic signal by phosphorylating the down stream target proteins such as the transcription factors, c-myc and c-jun, which control the expression of protein involved in growth (Lewis *et al.*, 1998). On the other hand, the p38 signal transduction pathway also plays an essential role in regulating such cellular processes as inflammation, differentiation, cell growth, and death (Iwasaki *et al.*, 1999).

In stomach cancer, expression of uPA/uPAR is essential for tumor cell invasion and metastasis. However, the mechanism that is responsible for uPA/uPAR expression in cancer cells remains unclear. It has been previously shown that ERKs plays an important role in controlling pancreatic cancer cell growth. Inhibition of the MEK/ERK cascade blocks pancreatic cancer cell proliferation while the activation of this pathway by growth factors and cytokines is associated with the induction of pancreatic cancer growth and invasion (Douziech *et al.*, 1999; Ehlers *et al.*, 2000). In addition, some studies have demonstrated that p38 MAP kinase negatively regulates pancreatic cancer cell proliferation (Xian *et al.*, 2001; Lee *et al.*, 2003a). In contrast, the role of ERK and p38 MAPK in regulating stomach cancer cell proliferation is not understood.

In this study, we will now try to show the interaction between the MEK/ERK and p38 MAP kinase pathways in human gastric cancer cells concomitant with the induction of the urokinase proteolysis network.

Materials and Methods

Cell culture

We used two human gastric cancer cell lines in our experiments: the poorly differentiated adenocarcinoma, NUGC-3, and the moderately differentiated tubular adenocarcinoma, MKN-28. The cell lines were obtained from the Korea Cell Line Bank. These cells were maintained on plastic in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, two-fold vitamin solution, and 50 U/ml penicillin/streptomycin (Life Technologies Inc., Gaithersburg, MD). Unless otherwise noted, the cells were passaged and removed when they were 70-80% confluent.

Western blot analysis

Cells were seeded onto six-well plates (1×10^6 cells/well) and incubated in a 5% CO₂ incubator for 24 h. The cells were then serum-starved for 24 h and treated with increasing concentrations or times

of HGF. The cells were harvested, then lysed in a lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% Glycerol, 1% Triton X-100, 1 mM Na₃VO₄, and 1 mM PMSF) and centrifuged at 13,000 $\times g$ for 5 min. The supernatants were transferred into new tubes and protein concentrations were determined using BCA protein assay reagent (Pierce Biotechnology Inc., Rockford, IL) using bovine serum albumin as a standard. The proteins (100 μ g) were separated in a 10% SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes, which were blotted with primary antibodies. Secondary antibodies conjugated with horseradish peroxidase were detected using chemiluminescence solution (KPL Inc., Gaithersburg, MD).

For uPA analysis, the cells (1×10^6 /well) were plated overnight in complete medium, starved for 24 h, and then treated with 5, 10 and 20 μ M MEK inhibitor (PD098059) or 1, 5 and 10 μ M p38 MAPK inhibitor (SB203580) for 1 h prior to incubation with 10 ng/ml HGF for 48 h. The uPA, secreted in media, was analysed by Western blotting using a rabbit polyclonal antibody against human uPA (389, American Diagnostica, Greenwich, CT).

Gel zymography for uPA

The cells were harvested. Equal amount of proteins were resolved under non-reducing condition in 10% SDS-polyarylamide gels impregnated with 0.4% casein and 15 mg/ml human plasminogen for uPA. After electrophoresis, the gels were washed for 2 h in a solution of 50 mM Tris-HCl (pH 7.5), 2% Triton X-100 and 0.02% NaN₃, and then they were placed overnight in 150 mM NaCl and 100 mM Tris solution. Following staining in a 0.2% Coomassie brilliant blue solution in methanol:acetic acid:water (4:1:5) and then destaining in a same solution without the dye, the enzyme activity was detected as the negatively stained regions. Zymographic analyses were performed in at least 3 independent experiments.

Anchorage-dependent cell proliferation by cell counting

The cells (1,000/well) were seeded in a 96-well plate in a medium supplemented with 5% FBS, and they were incubated for 24 h. Cells were then serum-starved for 24 h and treated for 72 h with or without HGF (10 ng/ml) in the presence of 5, 10 and 20 μ M PD098059 and 1, 5 and 10 μ M SB203580. At the end of this incubation period, 50 μ l of 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and the cells were allowed to incubate for 3 h at 37°C. The supernatant was carefully removed by aspiration and convert dye was dissolved with 100 μ l DMSO. The

plates were placed in a microplate shaker for 5 min, and the absorbance was measured at 570 nm using a Biorad multiscan plate reader.

Pre-treatment with PD098059 and SB203580

The cells (1×10^6 /well) were plated overnight in complete medium and then washed. The cells cultured in serum-free DMEM were pre-treated with 10 μ M PD098059 for 30 min, and then they were

treated or left untreated with 5 μ M SB203580 for 20 min. The cell lysates were immunoblotted with a phospho-ERK antibody.

Transient transfection

The dominant negative (DN) MEK1 (S218A, S222A) expression vector was kindly gifted from Dr. S.M. Ahn (Korean National Institute of Health, Seoul, Korea). The DN-MEK1 vector was transfected into

Figure 1. Effects of HGF on ERK activation. The cells were cultured in RPMI-10% FBS, washed and then incubated for an additional 24 h in serum free medium. The cells were then stimulated for 15 min with 0, 10, or 40 ng/ml HGF (A) or for 0, 1, 3, or 10, 30 min with 10 ng/ml HGF (B). Cell lysates were prepared and proteins were resolved on a 10% SDS-polyacrylamide gel under reducing condition. ERK1/2 activation was measured evaluated by Western blot analysis using ERK and phosphorylated ERK antibodies. Values are means $\pm$ SD of triplicates of three independent experiments.

NUGC3 cells using lipofectamine according to the manufacturer's protocol (Life Technologies Inc., Gaithersburg, MD). Cells were cultured in media containing 300 μ g/ml G-418 for 2 weeks. The G-418 resistant cell clones were selected and the level of ERK protein was analyzed by Western blotting.

Results

Activation of ERK and p38 kinase following treatment with HGF

To elucidate the involvement of MAPK activity on the HGF-mediated uPA expression, we analyzed the

Figure 2. Effects of HGF on the phosphorylation of p38 kinase. The Cells were cultured in RPMI-10% FBS and starved for 24 h in serum-free medium. The cells were then stimulated for 15 min with 10 and 40 ng/ml HGF. p38 activation was measured evaluated by Western blot analysis using p38 and phosphorylated p38 antibodies. Values are means $\pm$ SD of triplicates of three independent experiments.

ERK and p38 phosphorylation in the NUGC-3 cells, and the MKN-28 cell that had been treated with 0, 10 or 40 ng/ml HGF. ERK phosphorylation was increased in the HGF-treated cells in a dose dependent manner (Figure 1A). The addition of 10 ng/ml HGF to both the serum starved cell lines rapidly stimulated the ERK phosphorylation. ERK activation

occurred within 1-3 min and it declined by 30 min (Figure 1B). p38 phosphorylation was also increased in both the HGF-treated cell lines in a dose dependent manner (Figure 2A). p38 activity simultaneously increased with ERK activity (Figure 2B).

Figure 3. Effects of PD098059 and SB203580 on HGF-induced uPA expression in NUGC-3 cells. The cells were cultured in RPMI-10% FBS and starved for 24 h in serum-free medium. Serum-starved cells were pre-treated with 5, 10, or 20 μ M PD098059 and 1 or 5 μ M SB203580 for 1 h and then treated with 10 ng/ml HGF for 48 h. uPA expression in the supernatants was then analyzed by zymography (A) and Western blot analysis (B) under non-reducing conditions. Values are means $\pm$ SD of triplicates of three independent experiments.

Figure 4. Effects of PD098059 and SB203580 on cell proliferation. Cells were plated in 96 well plates at a density of 1.5×10^3 cells/well and starved in serum-free medium for 24 h. Cells were pre-treated with indicated concentrations of PD098059 or SB203580 for 72 h and then treated with 10 ng/ml HGF. Cell viability was measured by the MTT assay. Values are means $\pm$ SD of triplicates of three independent experiments.

Figure 5. Effects of PD098059 and/or SB203580 on HGF-induced ERK phosphorylation. Serum-starved cells were pre-treated with 10 μ M PD098059 and/or 1 and 5 μ M SB203580 for 30 min and then treated with 10 ng/ml HGF for 15 min. ERK1/2 activation was measured by Western blot analysis using ERK and phosphorylated ERK antibodies. Values are means $\pm$ SD of triplicates of three independent experiments.

Effects of PD098059 and SB203580 on uPA secretion

To test whether ERK activation and p38 activations were involved in the HGF-mediated uPA secretion, the cells were pre-treated with PD098059 or SB203580, and the uPA secretion was measured by Western blotting and gel zymography. The NUGC-3 cells showed that HGF-mediated uPA secretion was reduced with the increasing concentrations of PD098059. Densitometric analysis indicated that 20 μ M of the MEK1 inhibitor reduced the urokinase secretion by 40-50%. In contrast, pre-treatment with SB203580 increased the uPA secretion and activity. These results suggested that HGF-mediated uPA secretion and the augmentation of this activity were regulated by ERK and p38 activation (Figure 3).

Effects of PD098058 and SB203580 on cell proliferation

To determine whether MAP kinase plays a role in cell proliferation, MTT assay was performed after treatment of the cells with ERK, PD098058 and SB203580. Following 72 h treatment, PD098058 significantly blocked cell proliferation in a dose-dependent manner. In contrast, SB203580 had an effect on cell proliferation (Figure 4).

Effects of PD098059 and/or SB203580 on HGF-induced ERK1/2 phosphorylation

To investigate the possibility of interaction between the ERK and the p38 activations in the HGF-mediated uPA expression, the effects of SB203580 on ERK activation were measured. Pre-treatment of SB203580 increased ERK phosphorylation in the HGF-treated cells. A co-treatment of PD098058 and SB203580 decreased the ERK phosphorylation. These results suggested that the HGF-mediated uPA secretion and the augmentation of this activity were regulated by ERK activation, and p38 activation might indirectly affect HGF-mediated uPA secretion. In other words, the increments of HGF-mediated uPA secretion and its level of expression according to the treatment by SB203580 were mediated through the ERK activation (Figure 3 and 5).

Effects on a dominant negative-MEK1 gene on uPA expression

We transfected the dominant negative-MEK1 gene to NUGC-3 cells and measured the HGF-mediated uPA secretion. Transfection of DN-MEK1 caused a decrease in uPA secretion after stimulating with HGF, suggesting that the HGF-mediated uPA secretion was regulated by the ERK activation (Figure 6).

Figure 6. Effect of dominant-negative (DN) MEK1 on HGF-induced uPA expression in NUGC-3 cells. The DN-MEK1 was transfected into NUGC3 cells using lipofectamine and selected stable DN-MEK1 cells. ERK protein level was confirmed by Western blotting using an ERK antibody and equal loading of proteins was confirmed by a GAPDH antibody (A). Vector-transfected (control) cells and DN-MEK1 cells were starved for 24 h in serum-free medium and treated with HGF for 48 h. The uPA protein levels in the supernatants were then analyzed by Western blotting under non-reducing conditions (B).

Discussion

An increased expression of uPA has been associated with tumor invasiveness and metastasis. In addition, binding of uPA to its receptor can stimulate epithelial cell motility by a mechanism that is independent of proteolysis (Juan *et al.*, 2001). uPA expression can be regulated by growth factors that bind to tyrosine kinase receptors and this activates the Ras signaling pathway (Aguirre-Guiso *et al.*, 1999). This pathway involves the activation of MAPK by c-Raf, which in turn activates the extracellular signal regulated kinase (ERK).

Some studies have reported that hepatocyte growth factor and transforming growth factor- β_1 (TGF- β_1) stimulate the migration and invasiveness of transformed epithelial cells concomitantly with the up-regulation of uPA (Restuccia *et al.*, 2000; Lee *et al.*, 2003b). HGF's direct effects on cells for stimulating malignancy and for signaling through the HGF receptors very likely require c-Met. In a separate study, we have shown that HGF/c-Met signaling enhanced stomach cancer cell proliferation and it increased uPA synthesis and its activity. Inhibition of uPA receptors by a monoclonal antibody against the uPA receptor decreased tumor cell invasion.

The intracellular down stream signal for HGF/c-Met is still poorly understood, and several pathways might be involved. Transformed keratinocytes respond to TGF- β by their stimulated cell motility and invasiveness together with enhancement of the uPA

expression/secretion. Depletion of ERK protein by a treatment with antisense oligonucleotides reduced the basal uPA production and abolished any stimulation of uPA secretion and cell motility by TGF- β . We also demonstrated reduction of uPA production in NUGC-3 cell line transfected with DN-MEK1 gene. PD098059 decreased the TGF- β stimulated cell motility and invasiveness. These data suggest that the Ras/MAPK transduction pathway is involved in the invasive response of transformed keratinocytes to TGF- β (Matsumoto *et al.*, 1994). HGF also needs to cooperate with a *ras* oncogene for the progression of tumor cells to the invasive phenotype. The physiological role of ERKs for stimulating cell proliferation has been well documented. Constitutionally active MEK mutants and the elevated expression of MAP kinase have been reported to induce malignant transformation in adenocarcinoma and also in other cell types (Greulich *et al.*, 1998; Blalock *et al.*, 2000). ERK1/2 and p38 kinase act as critical downstream pathways that mediate the progression of stomach cancer cells. However, the specific downstream effects and physiological interactions of these proteins in stomach cancer cells are not fully understood. Our previous study showed that ERK and p38 kinase are involved in the HGF-mediated uPA expression in pancreatic cancer cell line, L3.6PL and IMIM-PC2.

Thus, we examined the role of the MAP kinase signaling pathway on the stimulation of uPA synthesis in stomach cancer cells by using HGF. This study showed that the phosphorylation of ERK and the p38 kinase are dependent on the dosage of HGF. We also showed that uPA secretion and zymoactivity in the NUGC-3 cell lines were stimulated with HGF, which suggests the involvement of ERK and p38 kinase in the HGF-mediated uPA expression.

The effects of PD098059 and SB203580 were measured in order to clarify which signaling pathway, between the ERK and p38 kinase pathways, might play the more important role in HGF-induced uPA secretion in the stomach cancer cell lines. While a pre-treatment with PD098059 reduced the uPA secretion, pre-treatment with SB203580 increased the uPA secretion. Increments of HGF-mediated uPA expression *via* SB203580 pre-treatment were also shown to be mediated by ERK activation. It is likely that a blockade on the ERK pathway affects the expression of multiple genes that are involved in the invasive process (Garbisa *et al.*, 1992; Crawford *et al.*, 1994; Matsumoto *et al.*, 1994). To determine whether ERK and p38 Map kinases play a role in cell proliferation, pre-treatment with PD098059 or SB203580 was done. SB203580 promoted proliferation of both cell lines in a concentration-dependent manner, but this proliferation was decrea-

sed by PD098059. Xian *et al.*, also reported similar results in the PCNC-1 pancreatic cancer cell line. They showed that SB203580 significantly enhances cell proliferation as measured by both thymidine incorporation and the cell number, indicating that p38 kinase functions as a negative growth regulator in the PANC-1 pancreatic cells. Further more, following a treatment with SB203580, more cells were remained in the S phase while there was a decrease of the number of cells in the G0/G1 phase, further supporting the importance of p38 MAP kinase as a negative regulator of PANC-1 cell proliferation (Xian *et al.*, 2001).

Based on this evidence, we have demonstrated that ERK and p38 MAP kinase are concurrently phosphorylated and activated in the NUGC-3 and MKN-28 stomach cancer cell lines that were first stimulated with HGF. Indeed, there is interaction between the p38 kinase and ERK pathways. This interaction was found to regulate uPA production in the carcinoma cells. The p38 kinase is a negative regulator for the ERK signaling pathway and hence, it negatively regulates stomach cancer cell proliferation and invasiveness. These findings raise the exciting possibility that PD98059 may be a clinical target for reducing the invasiveness of those tumors in which the signaling cascade is constitutively activated. In the future, we will perform studies aimed at evaluating the involvement of stress-activated MAPKs on stomach cancer cells' response to HGF.

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