Invasion-Metastasis by Hepatocyte Growth Factor/c-Met Signaling Concomitant with Induction of Urokinase Plasminogen Activator in Human Pancreatic Cancer: Role as Therapeutic Target

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Purpose: Increased expression of the hepatocytes growth factor (HGF) receptor (c-Met) and urokinase type plasminogen activator (uPA) correlate with the development and metastasis of cancers. However, the mechanisms by which HGF/c-Met signaling mediate cancer progression and metastasis are unclear. Therefore, we investigated the roles of HGF/c-Met in tumor progression and metastasis in pancreatic cancer cell lines, L3.6PL and IMIN-PC2.

Materials and Methods: To see the functional c-Met protein, we were performed immunoprecipitation for functional c-Met protein. And also performed western blot analysis and gel zymography for the functional uPA protein. To see the inhibition effects of uPAR monoclonal antibody on invasiveness of two pancreatic cancer cell lines, we were carried out standard two chamber invasion assay.

Results: At first, we observed the HGF-mediated c-Met phosphorylation and cell growth. c-Met phosphorylation was increased in the HGF-treated cells in a dose dependent manner. HGF resulted in increments of cell growth and ERK phosphorylation. HGF treatment increased the uPA expression and the uPA activity. A monoclonal antibody 3936, specific to uPAR receptor, inhibited HGF-mediated tumor cell invasion in a dose dependent manner.

Conclusion: These results suggest that functional c-Met and HGF/c-Met signaling up-regulate the activity of uPA and result in increments of invasion-metastasis in the pancreatic cancer cells. (Cancer Research and Treatment 2003;35:207-212)

Key Words: Metastasis, uPA, uPAR inhibition, ERK

INTRODUCTION

Hepatocyte growth factor/scatter factor (HGF/SF) is an effector of the cells expressing the Met tyrosine kinase receptor (1). HGF/SF is produced by mesenchymal cells and acts predominantly on the cells of epithelial origin in an endocrine and/or paracrine action. c-Met signaling has been reported to affect the status of cell to cell junction, integrin-matrix interaction, cytoskeletal organization and expression of motogenic protein (2). The molecular mechanism(s) by which HGF/c-Met signaling promotes the invasive-metastatic phenotype is largely unknown but probably involves the induction of proteases which mediate the degradation of the extracellular matrix-basement membrane. To date, emphasis has focused on the ability of c-Met ligands to coordinate up-regulate the expression of the serine proteasine, urokinase plasminogen activator (uPA) along with the surface-associated urokinase plasminogen activator receptor (uPAR) (3).

uPA is a serine protease of limited specificity. Active uPA is found predominantly at the cell surface. An over-expression of uPA has been reported for many malignant tumors including lung, breast, and colon cancer (4,5). Recent reports suggest that pancreatic cancer lesions with signs of invasion, exhibited the strongest immunohistochemical signal for uPA and uPAR in desmoplastic areas adjacent to the cancer cells. The marked overexpression of both factors may create an environment that enables pancreatic cancer cells to invade surrounding tissue (6). Because uPA can degrade, directly or indirectly, all components of the extracellular matrix, it has been hypothesized that this enzyme plays an important role in tumor invasion and metastasis. Previous studies have shown that antibodies, specific for urokinase, can inhibit metastatic dissemination of tumor cells in animal models (7). Furthermore, in vitro invasion assays on
cultured human tumor cells, demonstrate that uPA activity is essential for the invasive phenotype of these cells. uPAR is increasingly recognized as an important factor in focusing plasmin-mediated pericellular proteolysis. The amino-terminal domain of uPA is known to mediate uPA binding. uPAR binds uPA ligand with both high specificity and affinity (Kd $10^{-9}$ to $10^{-10}$ M) through the uPA growth factor-like domain (8). Resected human tumors have demonstrated the presence of the receptor on the malignant cells. Thus, it is becoming increasingly apparent that elevated urokinase receptor expression is a requirement for tumor invasion. This is an important issue to address with regard to developing novel anti-invasive and anti-metastatic agents that act by suppressing the production of the binding site.

Mitogen-activated protein kinase (MAPK) has been implicated in a variety of cellular functions, including growth factor-induced cell cycle progression (9). Extracellular-regulated kinases (ERKs) are activated by diverse extracellular agonists that promote cell growth (10). Activated ERKs subsequently transmit the mitogenic signal by phosphorylation factors that control expression of proteins involved in growth.

The goal of this study is to determine the action of HGF/c-Met signaling on the induction of uPA-mediated proteolysis and to determine its direct effects on the growth and invasion of pancreatic cancer. We will report on cellular mechanism regulating urokinase-type plasminogen-activation. Knowledge gained from this research may provide a therapeutic basis for interfering with metastasis.

**MATERIALS AND METHODS**

1) **Cell culture**

L3.6PL was kindly donated from Dr. Fidler (MD Anderson Cancer Center, Texas). IMIM-PC2 was kindly donated by Dr. Francisco X.R (Barcelona, Spain). L3.6PL and IMIM-PC2 pancreatic cancer cells were maintained on culture flasks in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, two-fold vitamin solution, and 50 U/mL penicillin/streptomycin (Gibco; Grand Island, NY). Unless otherwise noted, cells were passaged and removed from flasks when 70~80% confluent.

2) **Immunoprecipitation for functional c-Met protein**

Cells were plated with DMEM supplemented with 10% FBS and incubated for 24 h at 37°C and 5% CO₂. The cells were then serum-starved for 24 h followed by treatment with either HGF (0, 10, 40 ng/mL) for 15 minutes prior to lysis (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100, 1 mM Na₃VO₃, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20μM leupeptin, 0.15 U/ml aprotinin). The cell lysates were centrifuged at 12,000×g for 5 minutes at 4°C and the supernatants were transferred to a new tube. Protein concentration was measured using bovine serum albumin as a standard and a BCA Protein Assay Reagent (Pierce, Rockford, IL).

For c-Met immunoprecipitation, 200μg of cell lysate with 1μg of a mouse polyclonal antibody against c-Met (clone DO-24) (Upstate Biotechnology Inc; Lake Placid, NY) and protein A/G (Oncogene Research Products; Cambridge, MA) were incubated with rotation at 4°C overnight. After washing 3 times with a RIPA solution (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), the proteins were eluted with a SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2.5% b-mercaptoethanol, 0.01% bromophenol blue) by heating at 100°C for 5 min. The proteins were separated in a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Corp; Arlington, IL) and probed with an anti-phosphotyrosine antibody (clone 4G10, diluted 1 : 5,000) (Upstate Biotechnology Inc.), or anti-c-Met antibody (clone C-28, diluted 1 : 2,000, Santa Cruz Biotechnology; Santa Cruz, CA). Peroxidase-conjugated secondary antibodies were applied and immunoreactive proteins were visualized with an ECL solution (PerkinElmer Life Science, Boston, MA).

3) **Anchorage-dependant cell growth assay**

Cells (1,000/well) were seeded in a 96-well plate with a medium supplemented with 5% FBS and incubated for 24 h. Cells were then serum-starved for 24 h and treated for 72 h with 2% FBS, 2% FBS+HGF (40 ng/mL), or 10% FBS. At the end of this incubation period, 50μl of 2 mg/ml thiazol blue (MTT) solution was added and incubated for 3 h at 37°C. The supernatant was carefully removed by aspiration and converted formazan crystals were dissolved with 100 μl dimethyl sulfoxide. The plates were placed in a microplate shaker for 5 min and the absorbance was measured at 570 nm using a BioRad multispec plate reader.

4) **Western blot analysis and gel zymography for the functional uPA protein**

The cells (2×10⁵/well) were plated in a complete medium, washed, and treated with 0 or 40 ng/mL HGF, EGF, or IGF-I in L3.6PL and 0, 10, or 40 ng/mL of HGF in IMIM-PC2 for 48 h. uPA, secreted in media, was analyzed by Western blotting using a rabbit polyclonal antibody against human uPA (389, American Diagnostica, Greenwich, CT). The activity of uPA protein was detected by gel zymography. Equal amount of proteins were resolved under non-reducing conditions in a 10% SDS-polyacrylamide gel impregnated with 0.4% casein and 15μg/ml human plasminogen. Following electrophoresis, gels were washed for 2 h in a solution of 50 mM Tris-HCl, pH 7.5 containing 2% triton X-100, 0.02% NaN₃ and incubated overnight in a solution of 150 mM NaCl and 100 mM Tris-HCl, pH 7.5. Enzyme activity was detected as negatively stained regions following staining in 0.2% Coomassie blue solution in methanol : acetic acid : water (4 : 1 : 5) and destaining in the same solution without a dye.

5) **Standard two chamber invasion assay**

Cells at 80% confluency were detached in PBS containing 5 mM EDTA. Cells (1×10⁵) and uPAR mAb 3936 (0, 15, 25, or 50 ng/ml) were placed in the top of a Matrigel migration chamber with 0.8-micron pore (Fisher Scientific; Houston Texas). To the bottom chamber, media containing 5% FBS and HGF (0 or 40 ng/ml) with or without mAb 3936 (0, 15, 25, or 50 ng/ml) were added. Following incubation for 48 h, cells were then fixed and stained using a HEMA 3 stain set (Curtis Matheson Scientific; Houston, Texas) according to the manufacturer’s instruction. Cells migrated through the filter were quantitated by counting 10 fields at 200× power.
6) Western blot analysis for ERK1/2
L3.6PL and IMIM-PC2 cells were cultured on six-well plates until 1×10⁶ cells/well. Cells were then serum-starved for 24 h, followed by a treatment with HGF (0, 10, or 40 ng/ml) for 15 minutes prior to lysis. Cells were lysed in a lysis buffer (20 mM Tris base, 137 mM NaCl, 10% Glycerol, 1% Triton X-100, 1 mM NaVO₃, 1 mM PMSF) and centrifuged at 13,000 × g for 5 minutes. Supernatants were transferred into new tubes and the protein concentration was measured. The proteins (100 µg) were separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, and probed with anti-phosphoERK (T202/Y204, Cell Signaling) and anti-ERK antibodies (Cell Signaling).

RESULTS

1) HGF-mediated c-Met phosphorylation
Lysates from cells that were stimulated for 15 minutes in media with or without HGF were immunoprecipitated using an anti-c-Met antibody, resolved by SDS-PAGE, and subjected to Western blot analysis to determine whether L3.6PL and IMIM-PC2 cells harbored a functional c-Met protein. Cells showed an increase in c-Met specific autophosphorylation by HGF treatment as detected using anti-phosphotyrosine antibody (Fig. 1). Densitometric analysis demonstrated dose-dependent increments of c-Met phosphorylation. Cumulatively, these results demonstrate that L3.6PL and IMIM-PC2 cells have a functional c-Met protein, and that HGF exhibits an increasing capability to autophosphorylation of c-Met in the cells.

2) Anchorage dependent growth of L3.6PL cells in response to HGF
In order to determine whether HGF might affect cell proliferation, we performed an anchorage dependent growth assay using an MTT assay. After a 24 h incubation, the medium was removed, replaced with serum free DMEM and incubated with or without HGF. HGF-treated cells showed more than 20% increments of cell growth compared to the untreated cells (Fig. 2).

3) Induction of proteolysis of uPA following treatment with HGF
Since the invasive metastatic phenotype of cells could be strongly by the addition of exogenous HGF, the downstream effectors of this phenotype were examined, focusing initially on the proteolysis network. We investigated whether HGF affects the expression of uPA. uPA, secreted into media from cells that were exposed to HGF for 48 h, were analyzed by Western blotting using a uPA antibody. uPA expression in HGF-treated cells was 2.2 fold (L3.6PL) and 1.4 fold (IMIM-PC2) higher than in the untreated cells. However, IGF or EGF-treated L3.6PL cells showed a decrease in uPA expression compared to the untreated cells (Fig. 3). We measured the uPA activity using casein/plasminogen zymography in order to examine whether HGF-mediated uPA induction might be correlated with the uPA activity. As expected, HGF-mediated uPA induction

![Fig. 2](image-url). Effects of HGF on anchorage dependant cell growth. Cells (1,000 cell/well) were seeded in 96-well plate with medium supplemented with 5% FBS and adhered for 24 h. Cells were serum-starved for 24 h and treated for 72 h with or without HGF (0 and 40 ng/ml). And then 50 µl of a 2 mg/ml thiazol blue (MTT) solution was added. Following the incubation for 2 h at 37°C, the supernatant was carefully removed and convert dye was dissolved with 100 µl DMSO. Values are mean±SD of triplicates of three independent experiments and statistical significance was estimated by Student’s t-test (*: p<0.05; **: p<0.01).

![Fig. 3](image-url). Effects of HGF on uPA expression. Cells (2x10⁵/well) were grown in DMEM+10% FBS in six-well culture plates and then treated with 40 ng/ml of HGF, EGF, IGF for 48 h. uPA, secreted in the media, was analyzed by Western blotting using a rabbit polyclonal antibody against uPA.
Fig. 4. Casein zymography in cells cultured with or without HGF (10 and 40 ng/ml). uPA, secreted in the media, was resolved in a 10% SDS-polyacrylamide gel impregnated with 0.4% casein and 15 μg/ml human plasminogen. Enzyme activity was detected as negatively stained regions following staining with 0.2% Coomassie blue solution.

![Image](https://via.placeholder.com/150)

**Fig. 5.** Effects of HGF and mAb 3936 on the *in vitro* invasiveness of L3.6PL and IMIM-PC2 cells. Cells were placed in the top chamber of Matrigel-coated filter in media containing 5% FBS with or without mAb 3936 (0, 15, 25, or 50 ng/ml). The bottom chamber was filled with media containing 5% FBS and HGF with or without mAb 3936. After 48 h incubation, cells migrated through the filter were counted in 10 fields at 200× power. Values are mean ± SD of duplicates of three independent experiments and statistical significance was estimated by Student’s *t*-test (*: p<0.05; †: p<0.01).

was directly correlated with the increments of uPA activity. These results suggest that HGF/c-Met signaling results in the increased uPA expression as well as the increased uPA activity (Fig. 4).

4) Effects of HGF and uPAR mAb 3936 on cell invasion

To examine whether HGF/c-Met mediated uPA induction might affect invasive phenotype in the cells, we performed an *in vitro* invasion assay using a Matrigel migration chamber. In our data, the invasiveness in HGF-treated cells was more than 10 fold higher than in the untreated cells. If HGF-mediated uPA induction was involved in the invasive phenotype, blockade of uPA action might be the potential target for inhibiting tumor cell invasion. Therefore, we measured the effects of uPAR mAb 3936 on the tumor cell invasion. mAb 3936 showed a substantial reduction in HGF-mediated invasion in a dose dependent manner. These results suggest that HGF-mediated uPA induction affects the tumor cell invasion and the blocking of uPA action might be the potential therapeutic target for the metastasis (Fig. 5).

5) Activation of ERK following treatment with HGF

To elucidate the involvement of MAPK activity in HGF-mediated cell growth, we analyzed the ERK phosphorylation in cells treated with HGF. ERK phosphorylation was increased in HGF-treated cells in a dose dependent manner (Fig. 6).

**DISCUSSION**

The metastatic cells must complete a series of linked, sequential steps including detachment from the primary tumor, invasion into the surrounding tissue, penetration into target tissue and proliferation at the secondary site (11). The process of extracellular matrix (ECM) degradation is a continuously occurring process in several physiological processes such as cell migration, trophoblast implantation, ovulation, mammary gland involution, wound healing and in various pathological events including cancer invasion and metastasis. It is controlled by several proteases including uPA and matrix metalloproteases (12). Accumulating evidence from a series of basic and clinical studies have now shown the essential role a uPA system plays in promoting invasion and metastasis of several malignancies including breast, prostate and pancreatic cancer (12,13). In the absence of an autocrine component, tumor cells must derive HGF from the microenvironment through the stimulation of stromal cell compartment or through the action of proteases on ECM (14). The plasminogen activator is a serum protease that activates latent HGF *in vitro* due to HGF’s high sequence and structural similarity to plasminogen (15). The direct binding of Grb-2 to c-Met can initiate uPA gene transcription. Recent data indicates that HGF can upregulate uPA expression and uPA activity in human sarcoma and prostate cancer cells (6,16). The mechanism for this effect may involve uPA’s ability to both degrade ECM and activate latent proHGF. uPA expression has been documented in a number of human tumors where it is associated with poor prognosis and metastasis (17). In animal
models, the direct involvement of uPA was demonstrated through enhanced metastasis following uPA overexpression and through the inhibition of metastasis following treatment with uPA antagonist (18). Although HGF has previously been shown to enhance uPA activity in several cell types (19), to our knowledge, this is the first study to show the HGF-mediated uPA up-regulation and resultant augmentation of cellular invasion in the pancreatic cancer cells.

In addition to uPA, uPAR also plays important roles in metastasis. Malignant transformation of human fibroblasts correlated with increased levels of receptor-bound uPA activity (20). *In vitro* invasion by human glioblastoma cells could be modulated with an anti-uPAR antibody, blocking uPA binding to the receptor (21). In the tumor progression of human melanoma, neither uPA nor uPAR are detectable in benign or early stages but appear frequently in advanced primary melanoma and melanoma metastatic lesion (22). We found that mAb 3936 could reduce invasion of L3.6PL and 1MIM-PC2 cells in a dose-dependent manner and incubation of these cells with mAb 3936 (50 ng/ml) showed more than 50% inhibition. mAb 3936 was also reported to effectively block binding of uPA to uPAR on human M24 met melanoma cells (23) and glioblastoma cells (21). These data showed that although these pancreatic cancer cells secreted proteolytically active uPA, they could not effectively utilize uPA for invasion unless it might be bound to its receptor. A possible explanation for this phenomenon could be the increase of local concentration of uPA, as well as the enhancement of its proteolytic activity upon receptor binding. These processes would promote the generation of plasmin, hence, resulting in a more rapid ECM dissolution. Our findings demonstrated that uPAR was involved in at least one independent process, invasion, which is of potential importance for metastasis of human pancreatic cancer cells *in vivo*.

Studies of kidney epithelial cells overexpressing an activated c-Met demonstrated that uPA gene expression was activated through the MAP kinase pathway (24). There are at least three distinct MAP kinase signal transduction pathways in mammalian cells leading to activation of either the extracellular-regulated kinases (ERKs), the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) or the p38 MAPK. Growth factors and their receptors are involved not only in mitogenesis, but also in cell migration and invasion. In some systems, these processes are also dependent on activated MAPK (25). We attempted to analyze the activity of the ERKs in the human pancreatic cancer cell lines upon treatment with HGF. Our data showed that ERKs were activated in both cells by HGF, suggesting that the ERKs activation might be an essential signaling mechanism leading to cell invasion. Stimulation of cell invasion is a prerequisite for metastatic behavior of tumor cells. So we will identify the MAPK cascade as a signal transduction pathway involved in regulating tumor growth and metastasis in response to HGF in pancreatic cancer in the future.

**CONCLUSIONS**

Effects of the up-regulation of the uPA in response to HGF/c-Met signaling is the enhancement of invasiveness but other potential effects of uPA which may or may not influence invasiveness also deserve consideration. Further investigation will be necessary to determine the degree to which these unknown properties of HGF/c-Met signaling contribute to generation of the metastatic phenotype by this signaling pathway.

**REFERENCES**

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