IL-1β-stimulated urokinase plasminogen activator expression through NF-κB in gastric cancer after HGF treatment

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Abstract. The potential of hepatocyte growth factor (HGF) to regulate the expression of urokinase plasminogen activator (uPA) in a gastric cancer cell is not widely acknowledged. To identify the genes associated with the plasminogen activator proteolytic axis by HGF, we used cDNA microarray technology and selected genes upregulated or downregulated in two gastric cell lines (NUGC-3 and MKN-28). First, IL-1β RNA and protein were confirmed to be upregulated. Then, we investigated the effect of IL-1β induced by HGF on the uPA system, facilitating the migration and invasion of cancer cells in the metastatic process. The role for IL-1β in HGF-induced upregulation of uPA was determined by knockdown of IL-1β with IL-1β shRNA and a chromatin immune precipitation assay. The levels of IL-1β and uPA were upregulated in cells treated with HGF in a dose-dependent manner. HGF-induced upregulation of uPA was suppressed by IL-1β knockdown. HGF enhanced the binding activity of NF-κB to the uPA promoter in control cells, but not in the IL-1β shRNA cells. We confirmed the functional role of HGF inactivation of the uPA promoter by a reporter gene assay. Downregulation of IL-1β using IL-1β shRNA also decreased cell proliferation and invasion. IL-1β stimulated uPA expression through ERK and NF-κB in gastric cancer, which may therefore be promising targets for gastric cancer therapy.

Introduction

Gastric cancer is the second most common cause of cancer-related mortality (1,2), and the most common malignancy in Korea. Accumulated data have established that carcinogenesis is a multi-step process associated with alterations in cellular ontogenesis and tumor suppressor genes necessary for malignant transformation (3,4). Cancer cell metastasis and invasion are considered to represent uncontrolled tissue remodeling and degradation of the extracellular matrix (ECM) involved in the events of cancer progression (4,5). Several ECM degradation models involving matrix metalloproteinases (MMPs) and the plasminogen activators (PAs) proteolytic axis have been shown in various cell types (6). Tumor cell invasion and the metastatic process have been associated with elevated levels of cell surface urokinase plasminogen activator (uPA)-mediated plasminogen activation, while in vitro, in vivo and clinical studies have suggested that inhibition of cell surface uPA expression is associated with reduced tumor cell invasion, metastasis and improved clinical outcome (7,8).

Hepatocyte growth factor (HGF), which is produced by surrounding stromal cells, including fibroblasts and endothelial cells, has been shown to be a significant factor responsible for cancer cell invasion mediated by tumor stromal interaction. In our previous study, we reported that HGF increased the expression of uPA in gastric cancer cells (9).

Pro-inflammatory cytokines, such as IL-1, potently induce expression of proteases of the MMP and plasmin families in astrocytes in vitro (10,11). IL-1 gene polymorphisms are associated with the development of gastric atrophy and increased risk of gastric carcinoma. Several lines of evidence have shown that the concentration of IL-1β in plasma of patients with lung cancer is significantly elevated (12) and is linked to the risk of lung cancer (13). Moreover, IL-1β has been reported to regulate uPA expression in various cancer cells (14,15).

However, the mechanism by which IL-1β activates the metastatic phenotype in stomach cancer is unknown. Since uPA has a well-established role in tumor cell invasion and metastases, we undertook the present study to determine whether or not the expression of uPA is regulated by IL-1β, and to determine whether or not ERK and NF-κB are the predominant pathways for uPA regulation.

Materials and methods

Cell culture. We used two human gastric cancer cell lines [poorly differentiated adenocarcinoma (NUGC-3) and moder-
ately differentiated tubular adenocarcinoma (MKN-28)), which were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 2X vitamin solution, and 10 U/ml penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD, USA). Unless otherwise noted, cells underwent passage and were removed from flasks when 70-80% confluent.

Reagents and antibodies. The reagents and antibodies used in the experiments were purchased from the following sources: horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Bio-Rad Laboratories, Philadelphia, PA, USA); recombinant human HGF (R&D Systems, Inc., Minneapolis, MN, USA); rabbit polyclonal antibody against human IL-1β, Cell Signaling Technology, Inc. (Beverly, MA, USA); human recombinant HGF, Becton-Dickinson Lab (Beverly, MA, USA); uPA, American Diagnostica (Greenwich, CT, USA); NF-κB, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); PDTC, Calbiochem, Inc. (San Diego, CA, USA); PD098059, Biomol Research Laboratories, Inc. (Butler Pike, PA, USA) and LY294002 was from Calbiochem, Inc. Northern blot analysis. Total RNA was extracted by acid-phenol-guanidium thiocyanate-chloroform extraction. Total RNA (10 µg) was separated on a 1% formaldehyde agarose gel and transferred to a Hybond N+ nylon membrane by the capillary method. RNA was cross-linked by UV irradiation (1,400 µJ/cm^2) using a UV cross-linker (Uvp, Inc., Upland, CA, USA). The membrane was hybridized with a ^32P-labeled c-fos or c-jun probe overnight at 42°C, then washed in 2X SSC for 5 min at room temperature, 2X SSC/0.1% SDS at 42°C for 30 min, and 0.5X SSC/0.1% SDS at 42°C for 30 min. The membranes were exposed to X-ray films at -70°C. Equal loading of the RNAs was confirmed by hybridization with a ^32P-labeled GAPDH probe.

cDNA microarray analysis. The cDNA microarray containing a set of 17,448 sequence-verified human cDNA clones was provided by Genomictree, Inc. (Daejeon, Korea). cDNA microarray experiments were performed as described by Yang et al (16). Briefly, total RNA (100 µg) was reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP (25 mM stock; NEN Life Science Products, Boston, MA, USA) at 42°C for 2 h. The labeled cDNA was then hybridized with the cDNA microarray at 65°C for 16 h. The hybridized slides were washed, scanned with an Axon 4000B scanner (Axon Instruments), and analyzed using GenePix Pro 4.0 (Axon Instruments). Raw data were normalized and analyzed using GeneSpring 6.0 (Silicon Genetics). Genes were filtered based on intensity in the control channel. When the control channel values were <80 in all of the samples, we considered the results to be unreliable genes. Intensity-dependent normalization (LOWESS) was performed in which the ratio was reduced to the residual of the LOWESS fit of the intensity vs. ratio curve. Average normalized ratios were calculated by dividing the average normalized signal channel intensity by the average normalized control channel intensity. Welch's ANOVA test was performed for P-values ≤0.1 of 0.05 to identify sample genes differentially expressed. Correlation analysis was performed using Pearson correlation (-1 to 1). Spots showing changes ≥2-fold were considered significant.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA (cDNA) was synthesized from total RNA using MMLV reverse transcriptase (Promega Corp., Madison, WI, USA) by the oligo (dT) priming method in a 10 µl reaction mixture. PCR was performed in 10 µl reaction volume containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1 µl cDNA, 200 µM dNTPs, 1 mM MgSO_4, 1U of platinum Pfx Taq polymerase, and 2 µM primers. The reactions were as follows: the initial denaturation at 95°C for 4 min, 27 cycles at 94°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec and the final extension at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Western blot analysis. Cells were harvested and incubated with a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 1 mM sodium vanadate, and 5 mM NaF] with protease inhibitors and centrifuged at 15,000 rpm for 10 min at 4°C. Proteins (50 µg) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were soaked with 5% non-fat dried milk in TTBS [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20] for 30 min, then incubated overnight with a primary antibody at 4°C. After washing 6 times with TTBS for 5 min, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h 30 min at 4°C. The membranes were rinsed 3 times with TTBS for 30 min and the antigen-antibody complex was detected using an enhanced chemiluminescence detection system.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells and IL-1β shRNA (1,500/well) were seeded in 96-well plates in DMEM supplemented with 5% FBS and incubated for 24 h. Cells were then serum-starved for 24 h and treated for 72 h with or without HGF (10 ng/ml). At the end of this incubation period, 50 µl of a 2 mg/ml 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 µl of DMSO. The plates were placed in a microplate shaker for 5 min, and the absorbance was measured at 570 nm using a Biorad Multiskan plate reader.

IL-1β knockdown with short hairpin RNA (shRNA). The human IL-1β-specific shRNA expression vector (IL-1β shRNA, RH54333-NM_000576) containing an IL-1β-targeted shRNA sequence (AAACCCAGGGCTGGCTTGAGAG) was purchased from Open Biosystems (Huntsville, AL, USA). Cells were transfected with IL-1β shRNA using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD, USA). Clonal selection was conducted by culturing with puromycin (10 µg/ml) followed by serial dilution of the cells. Stable transfectant clones with low expression of the target genes were identified by western blot analysis.
Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology, Waltham, MA, USA) following the manufacturer's directions. Briefly, cells were fixed with 1% formaldehyde at 37°C for 10 min. Cells were washed twice with ice-cold PBS with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 mg/ml of aprotinin, and 1 mg/ml of pepstatin A), scraped and pelleted by centrifugation at 48°C. Cells were resuspended in a lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1)], incubated for 10 min on ice, and sonicated to shear DNA. After sonication, lysate was centrifuged for 10 min at 13,000 rpm at 48°C. The supernatant was diluted in ChIP dilution buffer [0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, and protease inhibitors]. Primary antibodies were added and incubated overnight at 48°C with rotation. The immunocomplex was collected by protein A/G agarose beads and washed with low-salt washing buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl (pH 8.1), and 150 mM NaCl], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl (pH 8.1), and 500 mM NaCl], LiCl washing buffer [0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)], and TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. The immunocomplex was then eluted using elution buffer (1% SDS, 0.1 M NaHCO₃, and 200 mM NaCl) and the cross-links were reversed by heating at 65°C for 4 h. After reaction, the samples were adjusted to 10 mM EDTA, 20 mM Tris-HCl (pH 6.5), and 40 mg/ml of proteinase K, and incubated at 45°C for 1 h. DNA was recovered and subjected to PCR amplification of the uPA promoter region (-1747 to -2042) using 5'-GAGGGGCGGCGAGGGGAGAA-3' (forward) and 5'-TGTGGTCAGTTTTGGATTTG-3' (reverse).

uPA promoter analysis. The transcriptional regulation of NF-κB by HGF was examined using transient transfection with a uPA promoter luciferase reporter construct (uPA-pMetLuc reporter). Cell transfection was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the luciferase reporter gene assay, control cells and shIL-1β expression cells were co-transfected with 1 µg of uPA-pMetLuc-reporter plasmids and 0.05 µg of phHYK plasmid, which was used as an internal transfection-efficiency control. Transfected cells were stimulated with or without 10 ng/ml of HGF for 1 h. The promoter activity was analyzed in each well of the cultured medium using a Dual Glo® luciferase assay system with a Turner Designs instrument luminometer (Turner Designs, Inc., Sunnyvale, CA, USA). The measured luminescence of firefly luciferase was divided by Renilla luciferase and the resulting quotient corresponded to the relative amounts of luciferase.

Statistical analysis. Values are expressed as means ± SD. The Student's t-test was employed for the analyses. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Induction of c-fos and c-jun by HGF in NUGC-3 and MKN-28 cells. As it is well-known that HGF induces c-fos and c-jun in a variety of cells, we tested whether or not NUGC-3 and MKN-28 cells also showed HGF-mediated c-fos and c-jun induction by northern blot analysis. As expected, the levels of expression of c-fos mRNA were increased with HGF at an early phase (to 30 min), then decreased in both cell lines; the c-jun mRNA levels gradually increased until 1 h after treatment with HGF in both cell lines (Fig. 1).

Identification of HGF-responsive genes by cDNA microarray analysis in NUGC-3 cells. In an attempt to explore differentially-expressed genes in NUGC-3 cells treated with HGF, we used 17k human cDNA microarrays. The initial analysis of the cDNA microarray expression data indicated that the presence of 36 genes changed by ≥2-fold after HGF treatment. A variety of genes were found to be differentially expressed. The genes were selected and the expressions were confirmed by RT-PCR. RT-PCR showed that the level of expression of IL-1β was enhanced with HGF.
increased after HGF-treatment (Fig. 2A). The level of IL-1β protein was also enhanced by HGF treatment, as confirmed by western blot analysis (Fig. 2B).

**Activation of IL-1β and uPA following treatment with HGF.** To elucidate the IL-1β and uPA expression following HGF treatment, we analyzed the expression after treatment with 0, 10 and 40 µg/ml of HGF in NUGC-3 and MKN-28 gastric cancer cell lines. IL-1β expression was increased in HGF-treated cells in a dose-dependent manner and uPA protein also was increased similar to IL-1β in a dose-dependent manner (Fig. 3).

**Effects of PD098059 and LY294002 on IL-1β and uPA expression.** To test whether or not ERK and PI3-kinase activation is involved in HGF-induced IL-1β and uPA expression, the cells were pre-treated with a MEK inhibitor (PD098059) or a PI3-kinase inhibitor (LY294002) and measured by western blotting. The cells showed that HGF-mediated IL-1β expression and uPA was decreased with PD098059. Densitometric analysis indicated that pre-treatment of PD098059 resulted in 2-4-fold decrements of IL-1β expression and 2-3-fold decrements of uPA expression in both cell lines. In contrast, pre-treatment of LY294002 showed no change in IL-1β and
uPA expression. These results suggested that HGF-mediated IL-1β and uPA expression is regulated by ERK, and not PI3-kinase (Fig. 4).

Effects of IL-1β on cell viability with IL-1β shRNA stable cells. To determine whether or not IL-1β plays a role in cell viability, we generated IL-1β shRNA stable cells. Knockdown IL-1β shRNA stable cells was confirmed by RT-PCR (data not shown). An MTT assay was performed after treatment of cells with HGF in both cell lines. Following 72 h of treatment, IL-1β had cell viability effects induced by HGF (Fig. 5).

Effects of IL-1β shRNA stable cells on regulation of uPA and a role of NF-κB. Following selection, cloning, and amplification of stable cells, we generated IL-1β stable cells. When the cell lines were treated with HGF to determine the effect of IL-1β knockdown cells on regulation of uPA and NF-κB, a decrease in IL-1β mediated uPA and NF-κB expression occurred in both gastric cancer cell lines (Fig. 6A). To assess the role NF-κB activity in HGF-mediated uPA regulation, we analyzed the level of expression of uPA after treatment with the NF-κB inhibitor, PDTC (100 mM). Expression of uPA was decreased in the PDTC treated cells (Fig. 6B).

Binding of NF-κB to a uPA promoter in both IL-1β shRNA cells and the luciferase reporter gene assay. We analyzed the promoter sequence of uPA genes to identify the putative NF-κB binding sequence using the TESS program (17). Two putative NF-κB binding sites were identified within the uPA promoter. The NF-κB transcription factor binding site for the uPA promoter was located within the proximal promoter regions upstream of the transcriptional start site (Fig. 7A). IL-1β shRNA-stable cells showed a decrease in the levels of IL-1β RNA as compared with control cells (Fig. 7B). To demonstrate a comparable NF-κB binding site function in the uPA promoter, IL-1β shRNA and control cells were treated with 10 µg/ml of HGF and binding of NF-κB to putative NF-κB binding sites was measured by the ChIP assay. HGF enhanced the binding activity of NF-κB to the uPA promoter with relatively strong constitutive activity in control cells, but not in the IL-1β shRNA cells (Fig. 7C). To further confirm the functional role of HGF in the activation of the promoter of genes identified by ChIP analysis, both cells were co-transfected with uPA promoter, then cultured with or without additional HGF, NF-κB inhibitor (PDTC). Knockout of the IL-1β gene decreased the basal and HGF-induced uPA promoter activity in both cells (Fig. 8). These findings provide direct evidence that the portion of the uPA promoter containing NF-κB sites is optional and activated by HGF-induced IL-1β.

Figure 5. Effects of IL-1β on cell viability. Cells (1,000/well) and stable IL-1β-shRNA cells were seeded in 96-well plates with DMEM media supplemented with 5% FBS and incubated for 24 h. After serum-starvation for 24 h, cells were treated HGF (10 ng/ml) for 72 h. Cell viability was measured by MTT assays and expressed as a percentage of HGF-untreated control cells. Values are the means ± SD of three independent experiments performed in triplicate. *P<0.05.

Figure 6. Effects of IL-1β on the expression of uPA and NF-κB in cells. Control cells and stable IL-1β-shRNA cells (1x10⁶/well) were plated overnight in complete medium, starved for 24 h, treated with or without 10 ng/ml HGF for 1 h and harvested. (A) The levels of expression of uPA and NF-κB were analyzed by western blotting. Effects of uPA on the NF-κB inhibitor, PDTC, in NUGC-3 and MKN-28 cells. The cells (1x10⁶/well) were plated overnight in complete medium, starved for 24 h, treated with or without 100 µM PDTC for 1 h and harvested. (B) The levels of expression of uPA were analyzed by western blotting. Representative data from three independent experiments are shown.
Effects of IL-1β knockdown cells on tumor invasion. To assess the role of IL-1β in HGF-mediated cell invasion, an in vitro invasion using a Matrigel migration chamber assay was used in both transfected cells. IL-1β shRNA cells had a decrease in HGF-mediated cell invasion compared to the control cells, suggesting that IL-1β may play an important role in HGF-mediated cell invasion through NF-κB and uPA (Fig. 9).

Discussion

The lack of a reduction in gastric cancer mortality rates indicates that there is an urgent need for the identification of novel targets that prevent or inhibit invasion and metastasis. Tumor invasion and metastasis are the major characteristics of aggressive phenotypes of various human cancers, and therefore the major causes of cancer mortality. Cancer cells must acquire several properties to disseminate from the primary
tumor, including the ability to degrade and migrate through the ECM, a process called invasion (18). Tumor invasion and metastasis are often associated with increased expression of ECM-degrading proteases, among which uPA is of central importance (19).

IL-1 is a potent pro-inflammatory cytokine that is upregulated in the presence of Helicobacter pylori and is important in initiating and amplifying the inflammatory response to infection (20,21). The IL-1 receptor antagonist (K-1ra) is a naturally-occurring anti-inflammatory cytokine that binds competitively to IL-1 receptors, thus blunting the potentially injurious effects of IL-1. Polymorphisms of IL-1β and IL-1 RN (encoding IL-1ra) have been reported to be associated with the risk for gastric carcinoma (22). Another report showed that the plasma level of IL-1β is significantly elevated in patients with lung cancer (12). Although uPA is regulated by cytokines, such as IL-1β, the intracellular signaling pathway leading to uPA expression remains largely unknown. Much effort has been directed at defining the signal transduction pathways induced by IL-1β. Several studies have documented that MAPKs have roles in IL-1β-induced signal transduction, but the profiles of IL-1β-induced kinase activation appear to vary in a cell type-dependent fashion (23).

Cheng et al (24) investigated whether or not IL-1β-induced expression of uPA was involved in lung cancer progression. In their study, IL-1β significantly induced uPA expression and activity via PKCα-dependent JNK1/2 and NIK cascades, linking to IKKα/β activation and p65 translocation and transcription activity using pharmacologic inhibitors and transfection with dominant-negative mutants and siRNAs. In an attempt to explore differentially-expressed genes in stomach cancer cells (NUGC-3) treated with growth factor, such as HGF, we found that IL-1β had a 3.26-fold upregulation using human cDNA microarrays. Although IL-1β-induced uPA regulation was similar to other studies (15,24), the mechanism by which the pathway appears to be the predominant pathway for uPA regulation was shown differently. Our results are consistent with the report showing that activation of p42/p44 MAPK is involved in the expression of uPA in different cell types (25,26). These differences may be due to cell types and different experimental conditions used in these assays. In addition, we found that pre-treatment with LY294002 could not enhance uPA expression. In the subsequent experiments, we characterized the sites in the uPA promoter that were required for IL-1β-induced uPA gene expression in NUGC-3 and MKM-28 cells.

As shown in Fig. 7, regions of the promoter containing candidate-binding sites for NF-κB at (-1880 to -1871, and -1857 to -1849) were required for IL-1β-mediated activation of the full-length (295 bp) uPA promoter. To further confirm the functional role of HGF in the activation of the promoter of uPA identified by ChIP analysis, we found that the portion of the uPA promoter containing NF-κB sites is optimal and is activated by HGF/IL-1β-induced NF-κB. This may be the first report to provide evidence that ERK plays an important role in the regulation of IL-1β-induced NF-κB activation in human gastric NUGC-3 and MKM-28 cells.

While the pathway by which tumor cells acquire invasive and metastatic capacity is probably cell type-dependent, little is known regarding this important biological process. The NF-κB transcription factor family is one of the major mediators of the intracellular function of IL-1β and the uPA promoter has NF-κB binding sites (27,28).

In our additional results, we reported that IL-1β induced IL-8 overexpression in IL-1β shRNA gastric cancer cell lines. Although it is well-known that IL-1β upregulates IL-8 expression in various cells, such as endothelial cells, epithelial cells, and smooth muscle cells (29), the molecular mechanism for IL-1β-induced IL-8 expression in gastric cancer is not known. Kitada et al (30) demonstrated that human gastric carcinomas overexpressed IL-8 and the IL-8 mRNA level directly correlated with the vascularity of the gastric tumor. The process of angiogenesis is essential for tumor growth. In the search for a better understanding of the process of tumor angiogenesis, it is necessary to acknowledge that the development of new blood vessels is dependent on the function and activity of tumor cells in the vascular microenvironment. Furthermore, HGF-induced upregulation of IL-8 might be more complex and could possibly be controlled by multiple transcriptional and/or post-transcriptional mechanisms. It is conceivable that a cytokine network exists between inflammatory cells producing cytokines that can initiate signaling in tumor cells.

In summary, we identified IL-1β-induced uPA expression via activation of the ERK1/2 and NF-κB pathways, which results in invasion of gastric cancer cells. These interactions might be promising targets for the development of future treatment strategies to improve the response rate and overall survival after treatment of gastric cancer.

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References


