

# EGF stimulates uPAR expression and cell invasiveness through ERK, AP-1, and NF- $\kappa$ B signaling in human gastric carcinoma cells

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**Abstract.** Overexpression of epidermal growth factor (EGF) and urokinase plasminogen activator receptor (uPAR) have been observed in human gastric cancers. However, the interaction between EGF and uPAR in gastric cancer has not been well elucidated. In this study, we investigated the effect of EGF on uPAR expression and the underlying signal pathways in human gastric cancer AGS cells. EGF induced uPAR mRNA expression in a time- and concentration-dependent manner. EGF also induced uPAR promoter activity. In addition, EGF induced the activation of extracellular signal regulated kinase-1/2 (ERK-1/2) and P38 mitogen-activated protein kinase (MAPK) but not the activation of c-Jun amino terminal kinase. A specific inhibitor of MEK-1 (an upstream effector of ERK-1/2) and a dominant negative MEK-1 were able to suppress the EGF-induced uPAR promoter activity. Site-directed mutagenesis and electrophoretic mobility shift assays demonstrated that the binding sites of transcription factors, activator protein-1 (AP-1) and nuclear factor (NF)- $\kappa$ B, are involved in the EGF-induced uPAR transcription. Suppression of the EGF-induced uPAR promoter activity by the AP-1 decoy oligonucleotide, as well as expression vectors encoding mutated-type NF- $\kappa$ B-inducing kinase and I- $\kappa$ B, confirmed that the activation of AP-1 and NF- $\kappa$ B are essential for the EGF-induced uPAR upregulation. The AGS cells pretreated with EGF showed a remarkably enhanced invasiveness and this effect was partially abrogated by uPAR neutralizing antibodies and by the inhibitors of ERK-1/2, AP-1, and NF- $\kappa$ B. The above results suggest that EGF induces uPAR expression via ERK-1/2, AP-1, and NF- $\kappa$ B signaling pathways and, in turn, stimulates cell invasiveness in human gastric cancer AGS cells.

## Introduction

Although the incidence of gastric cancer has decreased over the last few decades, it is still the most frequent digestive tract cancer and has a poor prognosis and a high mortality rate (1). Approximately 24,000 new cases of gastric cancer are diagnosed each year in the United States and ~700,000 worldwide (2,3). Due to local tissue invasion and metastasis, radiation therapy or chemotherapy does not significantly affect the length or quality of life of patients with advanced gastric cancers. An understanding of the detailed mechanisms of invasion and metastasis in gastric cancer would be helpful towards developing improved treatments.

Cancer invasion and metastasis are multifactorial processes and require the coordinated action of cell-secreted proteolytic enzymes and their inhibitors (4). Urokinase-type plasminogen activator (uPA), its inhibitors, and the uPA receptor (uPAR) form a complex proteolytic system that has been implicated in cancer invasion and metastasis. uPA, a serine protease, has the ability to convert plasminogen to active plasmin (5). Furthermore, the uPA-uPAR interaction can independently affect cell motility, integrin function, and gene expression (6). Overexpression of uPAR increases the ability of cells to penetrate a barrier of reconstituted basement membrane. Conversely, blockade of the uPAR by expression of a catalytically inactive enzyme or an antisense uPAR cDNA remarkably decreased the invasiveness of cells. In gastric cancers, increased levels of uPA and uPAR have been clearly demonstrated as essential to the maintenance of invasive and metastatic phenotypes, and these increases have been considered prognostically significant (7). The synthesis of uPAR is increased by a diverse set of agents including vascular endothelial growth factor, hepatocyte growth factor, fibroblast growth factor, transforming growth factor, okadaic acid, and phorbol ester (8,9).

Most gastric cancers overexpress epidermal growth factor (EGF) (10) and the activity of EGF has been identified as the key driver during the process of cell growth and replication. In addition to its effects on cell proliferation, there is some evidence that increased EGF expression may be related to migration and may also trigger epithelial cell signaling (11). However, the interaction between EGF and uPAR in gastric cancer has not been well elucidated.

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In this study, we found that EGF induced the expression of uPAR through the activation of the ERK-1/2, AP-1, and NF- $\kappa$ B signaling pathways, stimulating the invasiveness of human gastric cancer AGS cells.

## Materials and methods

**Cell culture and culture conditions.** Human gastric carcinoma AGS cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere with RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. The effects of EGF expression on uPAR were examined by harvesting the cells at different time intervals and measuring the level of uPAR mRNA via Northern blot analysis. In order to examine the role of specific signaling pathways during uPAR induction and increased cell invasiveness by EGF, the AGS cells were pretreated with 50  $\mu$ M PD98059 (a specific inhibitor of MEK-1, an upstream effector of ERK-1/2; New England Biolabs Inc., Beverly, MA, USA), 20  $\mu$ M SP600125 [(a c-jun N-terminal kinase (JNK) inhibitor; Calbiochem, San Diego, CA, USA)], 20  $\mu$ M SB203580 (a specific P38 MAPK inhibitor, Calbiochem), 40  $\mu$ M curcumin (an AP-1 inhibitor; Sigma, MO, USA), and 10  $\mu$ M BAY11-7082 (an NF- $\kappa$ B inhibitor) for 1 h prior to exposure to EGF.

**Northern blot analysis.** The extraction of total RNA and Northern blot hybridization were performed using the methodologies described previously (12). A cDNA probe was radiolabeled with [ $\alpha$ -<sup>32</sup>P]-deoxyribonucleoside triphosphate using the random priming technique with the Rediprime labeling system (Amersham Corp., Arlington Heights, IL, USA). The probed nylon membranes were exposed to radiographic film (Life Technologies Inc., Grand Island, NY, USA).

**Western blot analysis.** Protein extraction and Western blot analysis were performed as previously described (12). The primary antibody preparations used in this study were 1:1000 dilutions of rabbit polyclonal anti-phosphospecific ERK-1/2, anti-phosphospecific JNK, and anti-phosphospecific P38 MAPK antibodies (New England Biolabs Inc.). The secondary antibody was horseradish peroxidase-labeled anti-rabbit immunoglobulins from donkey (Amersham Corp.) used at a 1:3000 dilution. Protein bands were visualized using a commercially available chemiluminescence kit (Amersham Corp.). Total protein levels were assayed by washing the blotted membrane with stripping solution [100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl (pH 6.7)] for 30 min at 50°C and then reprobing the membrane with rabbit polyclonal anti-JNK and anti- $\beta$ -actin antibodies diluted at 1:1000.

**Measurement of uPAR promoter activity.** The transcriptional regulation of uPAR was examined by transient transfection of a uPAR promoter-luciferase reporter construct (pGL3-uPAR) (12). The plasmid pGL3/uPAR-promoter was kindly provided by Dr Y. Wang (Australian National University, Canberra, Australia) (13). AGS cells ( $5 \times 10^5$ ) were seeded and grown until they reached 60-70% confluence, then

pRLTK (an internal control plasmid containing the Herpes simplex thymidine kinase promoter linked to the constitutively active Renilla luciferase reporter gene) and pGL3-uPAR were cotransfected into the cells using FuGENE (Boehringer-Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. pRLTK was transfected as a negative control. Cells were incubated in the transfection medium for 20 h and treated with EGF for 2 h. The effect of signaling inhibitors on uPAR promoter activity were determined by pretreating cells with the inhibitors for 1 h prior to addition of EGF. The cotransfection studies were performed in the presence or absence of the AP-1 decoy oligodeoxynucleotides (ODNs) or MEK-1 (pMCL-K97M), I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\beta$ , or NF- $\kappa$ B-inducing kinase (NIK). The phosphorothioate double-stranded ODNs with the sequences against the AP-1 binding site (5'-CAGCTCAGAAAGTCACTTC-3', 3'-GAA GTGACTTCTGAGCTG-5') were prepared (Genotech Corp., Yuseong, Korea) and annealed (AP-1 decoy ODNs). The expression vector encoding the inactive MEK-1 (pMCL-K97M) was a gift from Dr N.G. Ahn (University of Colorado, Boulder, CO) (14). The dominant-negative mutants of I- $\kappa$ B $\alpha$  and I- $\kappa$ B $\beta$  (15) and NIK (16) were kindly provided by Dr D.W. Ballard (Vanderbilt University School of Medicine, Nashville, TN) and Dr W.C. Greene (University of California, San Francisco, CA), respectively. The importance of NF- $\kappa$ B and AP-1 during the induction of uPAR by EGF was examined by transfecting the AGS cells with pGL3-uPAR, in which the NF- $\kappa$ B and AP-1 site had been mutated.

**Extraction of nuclear proteins.** The 80-90% confluent AGS cells were incubated overnight in a medium containing 1% FBS and treated with 0-30 ng/ml EGF for 2 h. The cells were then resuspended in 500  $\mu$ l buffer A [50 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 3% (v/v) glycerol, and 1.5 mM MgCl<sub>2</sub>]. After allowing the cells to swell for 5 min on ice, they were lysed with 500  $\mu$ l of buffer B [identical to buffer A, except containing 0.05% Nonidet P-40 (Sigma, St Louis, MO, USA)]. The homogenate was gently layered onto a cushion of equal volume of buffer C [100 mM Tris (pH 7.4), 25% (v/v) glycerol, and 1.5 mM MgCl<sub>2</sub>] and centrifuged for 5 min at 200  $\times$  g. The white nuclear pellet was resuspended in 75  $\mu$ l of cold high-salt lysis buffer [20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride]. This suspension was agitated for 30 min at 4°C and then microcentrifuged for 15 min at 4°C. The resulting supernatant was stored in aliquots at -80°C. The protein was quantified spectrophotometrically using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

**Electrophoretic mobility shift assay (EMSA).** EMSA was performed with the Gel Shift Assay System (Promega). Briefly, the oligonucleotides containing the consensus sequence for AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') and NF- $\kappa$ B (5'-AGTTGAGGGGACTTTCCAGG-3') were end-labeled with [ $\alpha$ -<sup>32</sup>P] adenosine triphosphate (3000 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase and then purified in a Microspin G-25

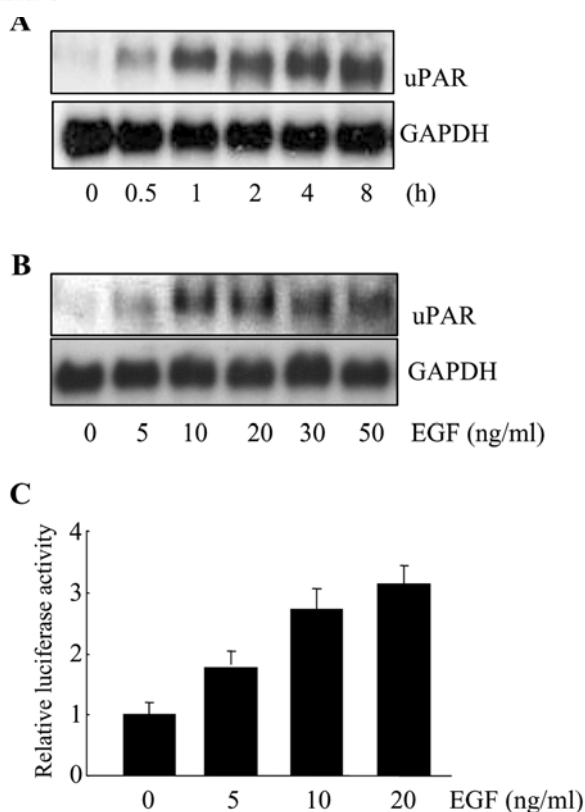


Figure 1. Induction of uPAR by EGF in human gastric cancer AGS cells. The AGS cells were incubated with 20 ng/ml EGF for 0-8 h (A) and 0-50 ng/ml for 4 h (B). After incubation, Northern blot analysis was performed. (C) AGS cells transiently transfected with pGL3-uPAR reporter construct. The transfected cells were incubated with 0-20 ng/ml EGF for 4 h and luciferase activity was determined using a luminometer. The data represent the mean  $\pm$  SD from triplicate measurements.

column (Sigma) and used as a probe for EMSA. Proteins from nuclear extracts (6  $\mu$ g) were pre-incubated with the binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 4% (v/v) glycerol, and 0.05 mg/ml poly(deoxyinosine-deoxycytosine)) for 5 min and then incubated with the labeled probe for 15 min at 37°C. Each sample was electrophoresed in a 5% non-denaturing polyacrylamide gel in 0.5x Tris borate-EDTA buffer at 150V for 4 h. The gel was dried and subjected to autoradiography.

**Matrigel invasion assay.** The cell invasion assay was carried out using Biocoat Matrigel invasion chambers (Becton-Dickinson, Bedford, MA, USA) according to the manufacturer's protocol. Of AGS cells 300  $\mu$ l ( $10^5$ ) was added to each chamber with 20 ng/ml EGF and allowed to invade the matrigel for 24 h. The number of invading cells was counted using phase-contrast microscopy. In order to determine the effect of the anti-uPAR antibody and inhibitors of ERK, AP-1, and NF- $\kappa$ B on the EGF-induced cell invasion, the AGS cells were preincubated with the neutralizing antibody against uPAR, 25  $\mu$ M PD98059, 40  $\mu$ M curcumin, and 10  $\mu$ M BAY11-7082 for 1 h and then added to 20 ng/ml EGF for 24 h.

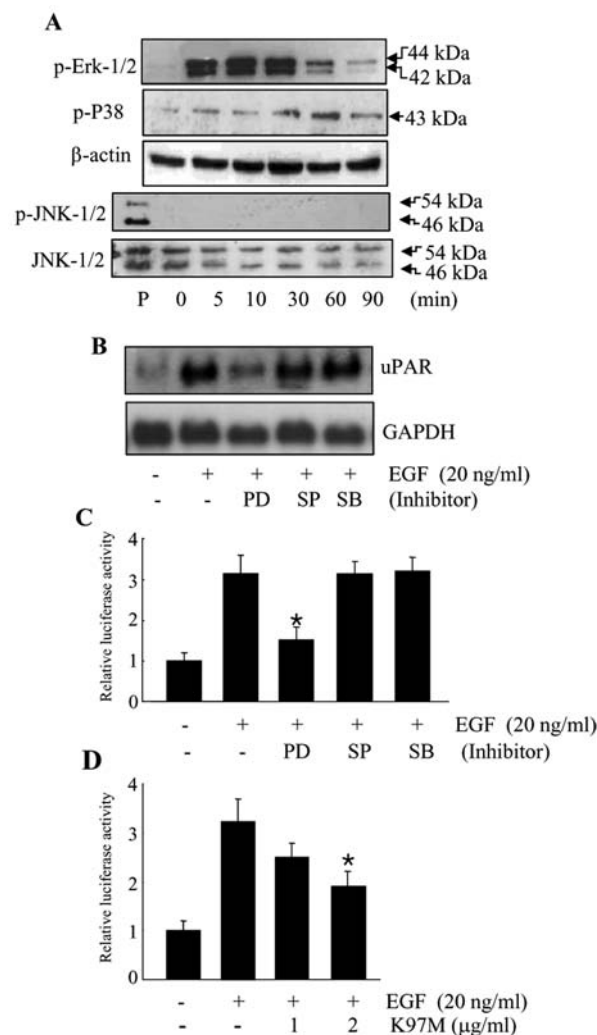


Figure 2. Involvement of ERK-1/2 during EGF-induced uPAR expression in human gastric cancer AGS cells. (A) AGS cells were incubated with 20 ng/ml EGF for 0-90 min, and cell lysates were assayed for the phosphorylated ERK-1/2, JNK-1/2, and P38 MAPK by Western blot analyses. The cells treated with 10 mM H<sub>2</sub>O<sub>2</sub> for 30 min were used as a positive control (P) for the phosphorylated JNK-1/2. (B) The AGS cells pretreated with 25  $\mu$ M PD98059 (PD), 20  $\mu$ M SP600125 (SP), and 10  $\mu$ M SB203580 (SB) for 1 h were then incubated with 20 ng/ml EGF for 4 h. After incubation, the levels of uPAR mRNA in the cell lysates were determined by Northern blot analysis (B). (C) The cells transiently transfected with pGL3-uPAR, after being pretreated with PD98059 (PD, 25  $\mu$ M), SP600125 (SP, 20  $\mu$ M), and SB203580 (SB, 10  $\mu$ M), were incubated with 20 ng/ml EGF for 4 h. After incubation, the cells were lysed and the luciferase activity was measured using a luminometer. (D) An expression vector encoding a mutated MEK-1 (K97M) was cotransfected with pGL3-uPAR into AGS cells. After incubation with 20 ng/ml EGF for 4 h, the luciferase activity was determined using a luminometer. The data represent the mean  $\pm$  SD from triplicate measurements. \*P<0.05 vs. EGF.

## Results

**Effect of EGF on uPAR mRNA levels and promoter activity in AGS cells.** To determine the effect of EGF on uPAR expression in AGS cells, cells were treated with EGF and the level of uPAR mRNA measured by Northern blotting. As shown in Fig. 1A, EGF induced uPAR mRNA expression in a time-dependent manner in gastric AGS cells. The uPAR mRNA expression increased appreciably 1 h after addition of

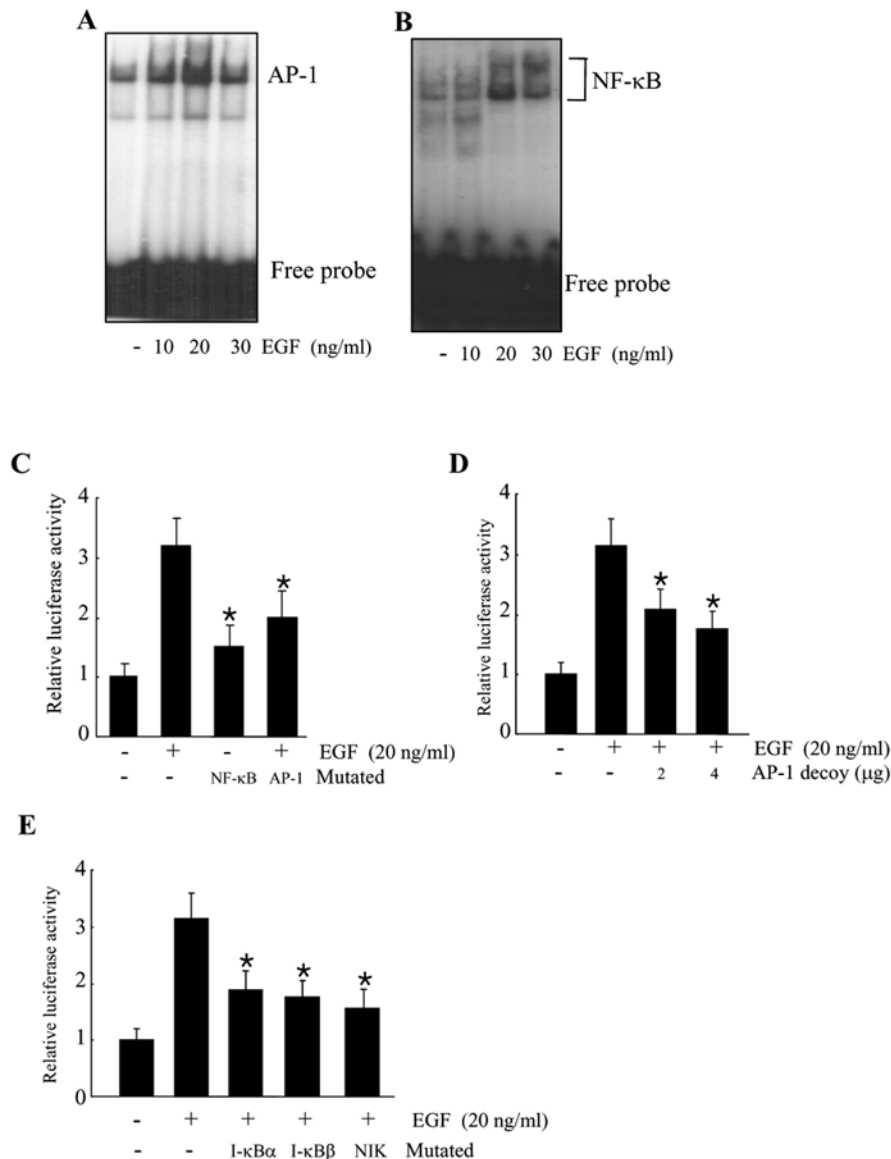


Figure 3. Activation of transcription factors AP-1 and NF- $\kappa$ B during uPAR expression by EGF. AGS cells were treated with 0-30 ng/ml EGF for 2 h (A and B). After incubation, the nuclear extracts from the cells were analyzed by EMSA for activated AP-1 and NF- $\kappa$ B using radiolabeled oligonucleotide probes. (C) Where indicated, the mutated pGL3-uPAR (with a converted AP-1 site TCCATGAGTCA to TCGGAATTCCA, and converted NF- $\kappa$ B site GGGAGGAGT to GGATCCAGT) was transfected into the cells. An AP-1 decoy oligonucleotide (AP-1 decoy, 0-4  $\mu$ g) (D) or dominant-negative mutants of I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\beta$ , and NIK (E) were cotransfected with pGL3-uPAR into the AGS cells. After incubation with 20 ng/ml EGF for 4 h, the luciferase activities were determined using a luminometer. The data represent the mean  $\pm$  SD from triplicate measurements. \* $P$ <0.05 versus EGF.

EGF to the cells. We also found that EGF induced the uPAR mRNA expression in a dose-dependent manner at 0-50 ng/ml (Fig. 1B). Next, we sought to examine the effect of EGF on transcriptional regulation of the uPAR gene. To this end, AGS cells were transiently transfected with the promoter-reporter construct (pGL3-uPAR) containing the human uPAR promoter and the luciferase gene. The AGS cells transfected with pGL3-uPAR showed an increase in promoter activity via EGF exposure in a dose-dependent manner (Fig. 1C).

**Involvement of ERK-1/2 in EGF-induced uPAR mRNA expression.** To study the signaling pathways involved in uPAR induction by EGF, changes in the levels of phosphorylated and total ERK-1/2, P38 MAPK, and JNK were determined in AGS cells after the cells were exposed to

EGF. As shown in Fig. 2A, EGF treatment led to an increased level of ERK-1/2 and P38 MAPK phosphorylation. However, EGF did not induce the phosphorylation of JNK. In order to examine the specific roles of MAPKs in EGF-induced uPAR expression, the AGS cells were pretreated with PD98059 (a MEK inhibitor), SB203580 (a P38 MAPK inhibitor), and SP600125 (a JNK inhibitor) before the EGF treatment. As shown in Fig. 2B, PD98059 partially blocked the EGF-induced expression of uPAR but SB20358 and SP600125 had no effect. To examine the role of ERK on transcriptional regulation of the uPAR gene, the AGS cells transfected with pGL3-uPAR were pretreated with PD98059, SB203580, and SP600125 before the EGF treatment. Consistent with Fig. 2B, only PD98059 inhibited the EGF-induced uPAR promoter activity (Fig. 2C). In addition, the EGF-induced uPAR



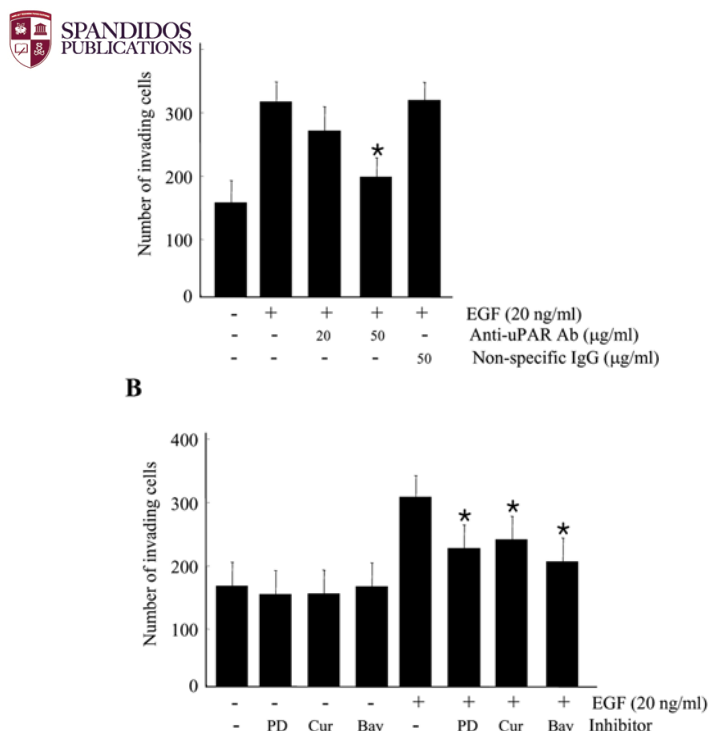


Figure 4. Effects of EGF and inhibitors on AGS cell invasiveness. AGS cells ( $10^5$ ) were incubated with 20 ng/ml EGF in a Boyden Matrigel for 24 h in the presence or absence of anti-uPAR antibodies (0-50  $\mu$ g/ml) (A), 25  $\mu$ M PD98059 (PD), 40  $\mu$ M curcumin, and 10  $\mu$ M BAY 11-7082 (B). After incubation, the cells having invaded the undersurface of the chambers were counted using a phase contrast light microscope after staining with a Diff-Quick stain kit. The data represent the mean  $\pm$  SD from triplicate measurements. \* $P < 0.05$  versus EGF.

promoter activity was inhibited in a dose-dependent manner when the expression vector encoding a mutated MEK-1 (K97M) was co-transfected with pGL3-uPAR into the AGS cells (Fig. 2D).

**Effect of EGF on the activation of transcriptional factors AP-1 and NF- $\kappa$ B during uPAR induction.** Since earlier studies suggested that the transcription factors NF- $\kappa$ B and AP-1 are essentially involved in uPAR expression in AGS cells (8,12,13), this study examined the effect of EGF on the activation of AP-1 and NF- $\kappa$ B. As shown in Fig. 3A and B, EGF treatment caused remarkable increases in the amount of AP-1 and NF- $\kappa$ B that could form a complex with the radio-labeled oligonucleotide probe in the EMSA. The involvement of the transcription factors AP-1 and NF- $\kappa$ B during the induction of uPAR by EGF were examined using the promoter-reporter construct (pGL3-uPAR). The AGS cells transfected with pGL3-uPAR with mutated AP-1 and NF- $\kappa$ B binding sites resulted in a decreased the EGF-induced uPAR promoter activity (Fig. 3C). Moreover, when the AGS cells were transfected with an AP-1 decoy oligonucleotide or dominant-negative mutant forms of I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\beta$ , and NIK along with pGL3-uPAR, a decreased EGF-induced uPAR promoter activity was observed (Fig. 3D and E).

**Effect of EGF on AGS cell invasiveness.** It has been suggested that expression of uPAR is required for the

invasive phenotype of cancer cells. To evaluate the role of EGF-induced uPAR during AGS cell invasion, cells were incubated with specific antibodies against uPAR in a modified Boyden invasion chamber. As shown in Fig. 4A, cell invasiveness was remarkably increased by incubation with EGF. However, the EGF-treated cells partially lost the increased Matrigel invasiveness after incubation with uPAR neutralizing antibodies, whereas they did not lose this Matrigel invasiveness after incubation with non-specific IgG. These results suggest that uPAR induced by EGF has an import role in gastric cancer cell invasiveness. In order to confirm that ERK-1/2, AP-1, and NF- $\kappa$ B are involved in the EGF-induced invasiveness, the AGS cells were treated with PD98059 (a MEK inhibitor), curcumin (an AP-1 inhibitor), and BAY11-7082 (an NF- $\kappa$ B inhibitor) before the EGF treatment. As shown in Fig. 4B, all of the inhibitors (PD98059, curcumin, and BAY11-7082) blocked the Matrigel invasiveness induced by EGF. However, the inhibitors alone did not significantly change the level of cell invasiveness. This suggests that the ERK-1/2, AP-1, and NF- $\kappa$ B signals activated by EGF upregulate uPAR, leading to an increase in gastric cancer cell invasiveness.

## Discussion

Many types of carcinoma cells including gastric cancers express EGF and EGFR at high levels, which have been implicated in tumor development and progression (10). The EGF and its receptor are not only critical for cell proliferation but also contribute to other processes that are crucial to cancer progression, including metastasis and angiogenesis (11). EGF has been shown to regulate tumor cell invasion through induction of many proteolytic enzymes, including matrix metalloproteinases (MMPs) and plasminogen activator (uPA) systems. Zhang *et al* (17) reported that NF- $\kappa$ B-mediated MMP-9 and uPA induction was responsible for EGF-induced invasiveness in pancreatic cancer cell lines. Henic *et al* (18) reported that an increase in the cell surface localization of uPAR in ovarian cancer cells resulted from increased expression of uPAR mRNA and decreased internalization and degradation of uPAR. Rosenthal *et al* (19) also reported that during EGF-induced invasion of the human squamous carcinoma cell line (UM-SCC-1), the cells expressed uPA and uPAR as well as MMPs, membrane-type MMP-1, collagenase 1, stromelysin 1, and gelatinase B. However, the induction of uPAR by EGF and its underlying molecular mechanisms in gastric cancer cells are not understood.

Our results show that EGF induced uPAR mRNA expression and promoted activation of ERK-1/2 and P38 mitogen-activated protein kinase (MAPK) in human gastric AGS cells. Recently, considerable effort has been directed at defining the signal transduction pathways induced by EGF in tumors. Cellular responses to EGF stimulation trigger a cascade of protein kinases that transmit signals from the cell surface to the nucleus, and these signal ultimately upregulate gene expression. Several studies have documented that the MAPKs have roles in EGF-induced signal transduction, but the profiles of EGF-induced kinase activation appear to vary in a cell type-dependent fashion. Three major MAPKs are

identified in mammalian cells: ERK-1/2, JNK, and P38 MAPK. In this study, we found that EGF induced uPAR expression and promoted the activation of ERK-1/2 in human gastric AGS cells. Increased ERK-1/2 and P38 MAPK phosphorylation was detectable within 10 min after exposure of the cells to 20 ng/ml of EGF. In contrast, JNK was not activated by the addition of EGF. Although, activation of ERK-1/2 and P38 MAPK preceded the induction of uPAR mRNA expression and this upregulation was attenuated by the selective inhibition of ERK-1/2 but not by inhibition of P38 MAPK. These results suggest that the ERK-1/2 signaling pathway is involved in the activation of the uPAR gene by EGF. This suggestion was further supported by the observation that expression of a vector encoding a mutated-type MEK-1 (K97M) resulted in a remarkable reduction in uPAR promoter activity. It remains unclear how EGF mediates ERK-1/2 activation in human gastric AGS cells. Wu *et al* (20) reported that EGF activates ERK-1/2 through the recruitment of Grb2, Shc, ErbB, and RAS. Activation of RAS leads to the activation of the MKKK, Raf-1. Raf-1 kinase then phosphorylates and activates the MKK, MEK1/2. Activated MEK1/2 then phosphorylates and activates ERK1/2. In addition, the generation of H<sub>2</sub>O<sub>2</sub> has been suggested as a common signaling event in MAPK activation via growth factors including EGF, basic fibroblast growth factor, and PDGF. Exogenously added H<sub>2</sub>O<sub>2</sub> was shown to elicit tyrosine phosphorylation in several cell types, whereas inhibition of peptide growth factors-induced increase in H<sub>2</sub>O<sub>2</sub> blocked various steps in signaling by these growth factors, including activation of MAPK (21).

In subsequent experiments, we characterized the sites in the uPAR promoter that were required for EGF-induced uPAR expression in AGS cells. Analysis of the genomic structure of uPAR reveals many potential targets for regulation at the transcriptional and post-transcriptional levels. Within its 3'-flanking region, the uPAR gene contains a single-copy of the AUUUA sequence, which is responsible for destabilization of various mRNAs (22). Within its 5'-flanking region, the uPAR gene contains the putative binding sites for activator protein-1 (AP-1), NF- $\kappa$ B, and TCF-1 but no potential TATA or CAAT boxes (13). As shown in Fig. 3C, site-directed mutagenesis indicated that the AP-1- and NF- $\kappa$ B-binding sites were required for activation of the uPAR promoter. Gel shift assays confirmed that EGF increased the DNA-binding activities of AP-1 and NF- $\kappa$ B. Our results are consistent with those of earlier studies implicating the involvement of AP-1 and NF- $\kappa$ B in the regulation of uPAR expression (8,12,13), although the relative contributions of these transcription factors appear to vary depending on the cell line and the stimulus used. The active AP-1 complex may be comprised of homodimers or heterodimers between c-Fos, c-Jun, and ATF2 (23). c-Fos activation can be regulated by the ERK and JNK signal pathways. Further, c-Jun is activated by N-terminal phosphorylation of specific serine residues (ser63/73) and it appears to be exclusively activated by JNK (24). Interestingly, treatment with ERK-1/2 inhibitors prevented the activation of AP-1, but not that of NF- $\kappa$ B (data not shown). Therefore, it is suggested that AP-1 is a downstream target of the MAPK pathways during the process of EGF-induced uPAR

expression. A similar observation was made by Kajanne *et al* (25), who observed that the ERK pathway and AP-1 are downstream effectors of the EGF-mediated MMP-3 expression and collagen contraction in fibroblasts. The signaling pathway leading to NF- $\kappa$ B activation by EGF receptor binding remains controversial. Habib *et al* (26) reported that EGF uses mechanisms similar to TNF, in that it recruits a distinct signalosome for the activation of NF- $\kappa$ B. EGFR binding to its ligand recruits the TNFR-interacting protein and NF- $\kappa$ B-inducing kinase.

Understanding the mechanism by which the expression of uPAR is regulated by EGF may lead to the development of new strategies for the treatment of gastric carcinomas, and further studies will be required to elucidate the detailed regulatory mechanism.

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