

# Targeting of Urokinase Plasminogen Activator Receptor in Human Pancreatic Carcinoma Cells Inhibits c-Met- and Insulin-like Growth Factor-I Receptor-Mediated Migration and Invasion and Orthotopic Tumor Growth in Mice

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## Abstract

**Pancreatic carcinomas express high levels of urokinase-type plasminogen activator (uPA) and its receptor (uPAR), both of which mediate cell migration and invasion. We investigated the hypotheses that (a) insulin-like growth factor-I (IGF-I)- and hepatocyte growth factor (HGF)-mediated migration and invasion of human pancreatic carcinoma cells require uPA and uPAR function and (b) inhibition of uPAR inhibits tumor growth, retroperitoneal invasion, and hepatic metastasis of human pancreatic carcinomas in mice. Using transwell assays, we investigated the effect of IGF-I and HGF on L3.6pl migration and invasion. We measured the induction of uPA and uPAR following treatment of cells with IGF-I and HGF using immunoprecipitation and Western blot analysis. The importance of uPA and uPAR on L3.6pl cell migration and invasion was studied by inhibiting their activities with amiloride and antibodies before cytokine treatment. In an orthotopic mouse model of human pancreatic carcinoma, we evaluated the effect of anti-uPAR monoclonal antibodies with and without gemcitabine on primary tumor growth, retroperitoneal invasion, and hepatic metastasis. IGF-I and HGF mediated cell migration and invasion in L3.6pl cells. In addition, IGF-I and HGF induced uPA and uPAR expression in L3.6pl cells. *In vitro*, blockade of uPA and uPAR activity inhibited IGF-I- and HGF-mediated cell migration and invasion. Treatment of mice with anti-uPAR monoclonal antibody significantly decreased pancreatic tumor growth and hepatic metastasis and completely inhibited retroperitoneal invasion. Our study shows the importance of the uPA/uPAR system in pancreatic carcinoma cell migration and invasion. These findings suggest that uPAR is a potential target for therapy in patients with pancreatic cancer. (Cancer Res 2005; 65(17): 7775-81)**

## Introduction

Pancreatic adenocarcinoma is characterized by aggressive invasion and metastasis. The 5-year overall survival rate for all

patients with pancreatic cancer is only 4%, which is essentially unchanged from two decades ago despite intensive research (1). Even for patients with resectable disease at presentation, the 5-year overall survival rate is only 21% (2). More effective therapies for pancreatic carcinoma are thus needed.

An understanding of the mediators of the critical steps in the metastatic process is essential to the development of targeted therapies for pancreatic carcinoma. Tumor cells must invade through the adjacent basement membrane into surrounding tissues and then migrate to and invade the vasculature to metastasize to distant sites. The processes of tumor cell invasion and migration involve a dynamic interaction between the tumor cells and the extracellular matrix. Migration and invasion are regulated by multiple cytokines and growth factors, integrins, matrix-degrading enzymes, loss of activity of degradative enzyme inhibitors, and cell-cell adhesion molecules/communication (3). Two growth factors in particular, insulin-like growth factor-I (IGF-I) and hepatocyte growth factor (HGF)/scatter factor (hereafter referred to as HGF), are potent mediators of pancreatic carcinoma cell migration and invasion (4, 5).

The urokinase-type plasminogen activator (uPA)/uPA receptor (uPAR) system has also been shown to mediate cell migration and invasion. uPA, a 50 kDa glycoprotein, is initially secreted as an inactive proenzyme and is activated by proteolytic cleavage. uPAR is a glycolipid-anchored receptor for uPA with multiple functions (6, 7). Activation of uPAR by uPA binding results in activation of the Ras/extracellular signal-regulated kinase pathway (also known as the mitogenic pathway), which leads to cell proliferation, migration, and invasion (8, 9). uPA converts plasminogen to plasmin, which facilitates matrix degradation, and activates several matrix metalloproteinases (10, 11). uPAR associates with integrins and controls their activation, which contributes to migration and invasion (12–14). Additionally, vitronectin, a component of the extracellular matrix, binds to uPAR and subsequently activates the small GTPase Rac1 (15, 16), which has been shown to regulate cell proliferation and migration (17).

Because of their role in the processes outlined above, uPA and uPAR are important mediators of tumor progression and metastasis. Expression of uPA and uPAR has been shown in many tumor types including glioblastoma and prostate, renal cell, breast, colon, hepatocellular, and pancreatic cancers (5, 18–20). Furthermore, higher levels of uPA and uPAR have been shown to correlate with poor prognosis in various malignancies (21–26), including pancreatic carcinoma (27, 28). Inhibition of uPAR in preclinical models led to increased glioblastoma cell death (29) and decreased

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glioblastoma and colon carcinoma cell invasion *in vitro* (30, 31). *In vivo* inhibition of uPAR resulted in a decrease in orthotopic growth and angiogenesis of breast carcinoma (32), inhibition of epidermoid carcinoma cell intravasation (33), and decreased lung metastasis formation following i.v. injection of colon carcinoma cells (34) and human fibrosarcoma cells in mice (35). However, the role of uPA or uPAR in regulating the growth, migration, and invasive properties of human pancreatic carcinomas remains to be determined.

In the current study, we investigated the hypotheses that (a) IGF-I- and HGF-mediated migration and invasion of human pancreatic carcinoma cells require uPA and uPAR and (b) inhibition of uPAR inhibits primary tumor growth, local invasion, and hepatic metastasis of human pancreatic carcinomas grown orthotopically in mice. *In vitro*, we found that IGF-I- and HGF-mediated migration and invasion of human pancreatic carcinoma cells required downstream activation of the uPA/uPAR system. We also found that treatment of mice with a monoclonal antibody (mAb) that inhibits uPAR significantly reduced orthotopic tumor growth and liver metastasis and completely inhibited retroperitoneal invasion.

## Materials and Methods

**Cell lines and cell culture conditions.** The human pancreatic carcinoma cell line L3.6pl (36) was kindly provided by I.J. Fidler, D.V.M, Ph.D. (The University of Texas M.D. Anderson Cancer Center, Houston, TX). Cells were cultured and maintained in MEM supplemented with 10% fetal bovine serum (FBS), 2 units/mL of a penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD), vitamins (Life Technologies, Inc., Grand Island, NY), 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and nonessential amino acids and incubated in 5% CO<sub>2</sub>/95% air at 37°C.

**Transwell migration and invasion assays.** To assess cell migration *in vitro*, L3.6pl cells ( $1.5 \times 10^5$  cells in 500  $\mu$ L MEM supplemented with 1% FBS) were placed in the top chamber of transwell migration chambers (8  $\mu$ m BioCoat Control Inserts, Becton Dickinson Labware, Bedford, MA). The lower chamber was filled with 750  $\mu$ L MEM supplemented with 10% FBS plus one of the following: (a) PBS, (b) recombinant human IGF-I (100 ng/mL; R&D Systems, Minneapolis, MN), (c) recombinant human HGF (100 ng/mL; R&D Systems), or (d) IGF-I plus HGF (100 ng/mL). After 24 hours, unigrated cells were removed from the upper surface of the transwell membrane with a cotton swab, and migrated cells on the lower membrane surface were fixed, stained, photographed, and counted under high-power magnification.

To assess invasion, *in vitro* invasion assays were done under the same conditions as the transwell migration assays, but in Matrigel-coated transwells (BioCoat Matrigel Invasion Chamber, Becton Dickinson Labware).

**Immunoprecipitation and Western blot analysis of urokinase-type plasminogen activator and urokinase-type plasminogen activator receptor.** To evaluate the effect of IGF-I and HGF on induction of uPA expression, L3.6pl cells at 80% confluency were incubated with PBS, IGF-I, HGF, or IGF-I plus HGF (100 ng/mL each). After 24 hours, conditioned media were collected, concentrated with a Centricon YM-3 centrifugal filter (Millipore Corp., Bedford, MA) at  $3,000 \times g$  for 50 minutes, and subjected to Western blot analysis for determination of uPA protein expression, as previously described (37). To determine the effect of IGF-I and HGF on induction of uPAR expression, cells were treated as above, cell lysates were collected and immunoprecipitated with anti-uPAR antibodies, and lysates were subjected to Western blot analysis for determination of uPAR protein expression, as previously described (38).

**Characterization of anti-urokinase-type plasminogen activator receptor (ATN-658) and anti-urokinase-type plasminogen activator (ATN-292) monoclonal antibodies.** ATN-658 was raised against a chymotryptic fragment of soluble uPAR expressed in *Drosophila* S2 cells using standard techniques. Briefly, BALB/c mice were immunized with

soluble uPAR fragments conjugated to keyhole limpet hemocyanin and the magnitude of the immune response was monitored by ELISA. Based on these data, hybridomas were generated by fusing spleen cells with the myeloma cell line P3x63Ag8.653. Frozen stocks of 10 parental hybridomas were made and five of the hybridomas were subjected to limiting dilution. Tissue culture supernatants from these monoclonal antibodies were then assayed for activity in ELISA assays and the isotype of each antibody determined using IsoStrips (Roche, Indianapolis, IN). ATN-658, isotype immunoglobulin G (IgG)-1 $\kappa$ , bound soluble uPAR immobilized to plastic with a  $K_D$  of  $\sim 1$  nmol/L, and iodinated ATN-658 specifically bound ATN-658 on the surface of HeLa cells with a  $K_D$  of  $\sim 5$  nmol/L. Western blot analysis showed that ATN-658 was specific for human uPAR and did not cross-react with mouse uPAR. For *in vivo* studies, ATN-658 was purified from tissue culture supernatant by column chromatography using protein A-Sepharose, typical yields ranging from 60 to 120 mg/L of tissue culture supernatant, and the purity of the final material was >95% as determined by high-performance liquid chromatography. Similarly, monoclonal antibody ATN-292 was raised against the NH<sub>2</sub>-terminal fragment of urokinase expressed in *Drosophila* S2 cells and conjugated to keyhole limpet hemocyanin. ATN-292, isotype IgG1 $\kappa$ , bound immobilized scuPA with a  $K_D$  of  $\sim 1$  nmol/L and competed the binding of iodinated NH<sub>2</sub>-terminal fragment to HeLa cells with an IC<sub>50</sub> of  $\sim 2$  nmol/L. For *in vivo* studies, ATN-292 was purified from ascites fluid by column chromatography using protein A-Sepharose as described above.

**Assays of urokinase-type plasminogen activator and urokinase-type plasminogen activator receptor inhibition.** The uPA inhibitor amiloride (100  $\mu$ mol/L; Sigma-Aldrich Co., St. Louis, MO; ref. 39) was added to L3.6pl cells in the upper chamber of transwells at a final concentration of 100  $\mu$ mol/L to study the effect of uPA inhibition on migration and invasion. Because amiloride has the potential to inhibit other enzymes owing to its lack of specificity at high doses, migration assays were repeated with specific blocking mAbs to uPA and uPAR, using isotype-controlled, nonspecific IgG as a control. The anti-uPA mAb, anti-uPAR mAb, or IgG was added to cells in the upper chamber of transwells at 10 ng/mL 2 hours before treatment with IGF-I and HGF.

**Cell survival as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done to evaluate the survival effects of IGF-I, HGF, and IGF-I plus HGF on L3.6pl cells and ensure that any increase in migration or invasion was not simply due to an increase in cell number. Assays were done with and without pretreatment with amiloride for 2 hours to assess the effect of amiloride on cell proliferation. Cells ( $1.5 \times 10^5$ ) were plated in 96-well plates in 1% FBS-containing MEM (initial conditions in upper chamber of transwells) or 6% FBS-containing MEM (representing equilibrated conditions between the upper and lower chambers of the transwells); PBS, IGF-I, HGF, or IGF-I plus HGF was added to achieve a final concentration of 100 ng/mL. Assays were done in quadruplicate. After 24 hours, 2 mg/mL MTT (Sigma-Aldrich) in PBS was added (50  $\mu$ L/well), and plates were incubated at 37°C for 2 hours. Media and MTT were removed, DMSO was added for 10 minutes, and absorbance was measured at 570 nm. For each of the MTT assays, the cells were kept under the same culture conditions, plated at the same cell density, and treated for the same time period with the same dose of IGF, HGF, or amiloride to replicate the conditions of the migration and invasion assays.

**Orthotopic tumor model.** L3.6pl cells were grown to 80% confluency and trypsinized, and cell viability was confirmed by trypan blue exclusion. Cells ( $1 \times 10^6$  cells/mouse) were injected into the tail of the pancreases of anesthetized 8-week-old male nude mice as previously described (40). Mice were housed and maintained in accordance with the standards of The University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee. Fifty mice were then randomized to one of the following four treatment groups: (a) IgG plus saline, (b) IgG plus gemcitabine, (c) anti-uPAR mAb plus saline, and (d) anti-uPAR mAb plus gemcitabine. Gemcitabine (15 mg/kg) and antibodies (10 mg/kg) were given i.p. twice per week beginning on day 4, and treatment continued until the mice were sacrificed on day 28. Sixteen hours before sacrifice, mice were injected i.p. with 0.2 mL (50 mg/mL) 5-bromo-2'-deoxyuridine (BrdUrd; Upstate, Lake Placid, NY).

During necropsy, the mice were evaluated grossly for evidence of tumor invasion into the retroperitoneum. Tumors were excised and measured with calipers and tumor volume was calculated using the following formula: volume =  $1/2 \text{ length} \times (\text{width})^2$ . Tumors were processed for paraffin embedding and frozen sections. Livers were excised and analyzed for the presence of surface metastases.

**Tissue staining and immunohistochemistry.** Paraffin-embedded sections of the primary pancreatic tumors were stained with H&E by standard methods. The presence of retroperitoneal invasion was histologically confirmed by noting invasion of the tumor through the peritoneal encasement of the peripancreatic tissue. H&E-stained sections were evaluated under high-power magnification for differences in percent area of tumor necrosis.

Paraffin-embedded sections were evaluated for apoptosis by staining with antibodies to activated caspase-3 (PhenoPath Laboratories, Seattle, WA). Tumor microvascular density and tumor vessel area were analyzed by staining frozen sections with anti-CD31 antibodies, as previously described (40). Immunohistochemical staining was analyzed with Scion Image software (Scion Corp., Frederick, MD). The number of anti-activated caspase-3 stained cells per high-power field was measured. Microvascular density was calculated as the number of tumor vessels per high-power field, and the tumor vessel area was calculated as the percentage of tumor area containing blood vessels.

To evaluate the effect of treatment on tumor cell proliferation, frozen sections were stained with anti-BrdUrd antibodies, and the number of BrdUrd-positive cells per high-power field was analyzed as previously described (41).

**Statistical analyses.** Statistical analyses were done using InStat 3.01 software (GraphPad Software, Inc., San Diego, CA). The significance of differences between treatment groups and control groups was determined using the Mann-Whitney *U* test, Student's *t* test, or Fisher's exact test, as appropriate. Significance was determined with 95% confidence.

## Results

### Effect of Insulin-like Growth Factor-I and Hepatocyte Growth Factor on Pancreatic Carcinoma Cell Migration and Invasion

**Migration.** To evaluate the effect of IGF-I and HGF on L3.6pl cell migration, transwell migration assays were done. IGF-I led to a mean

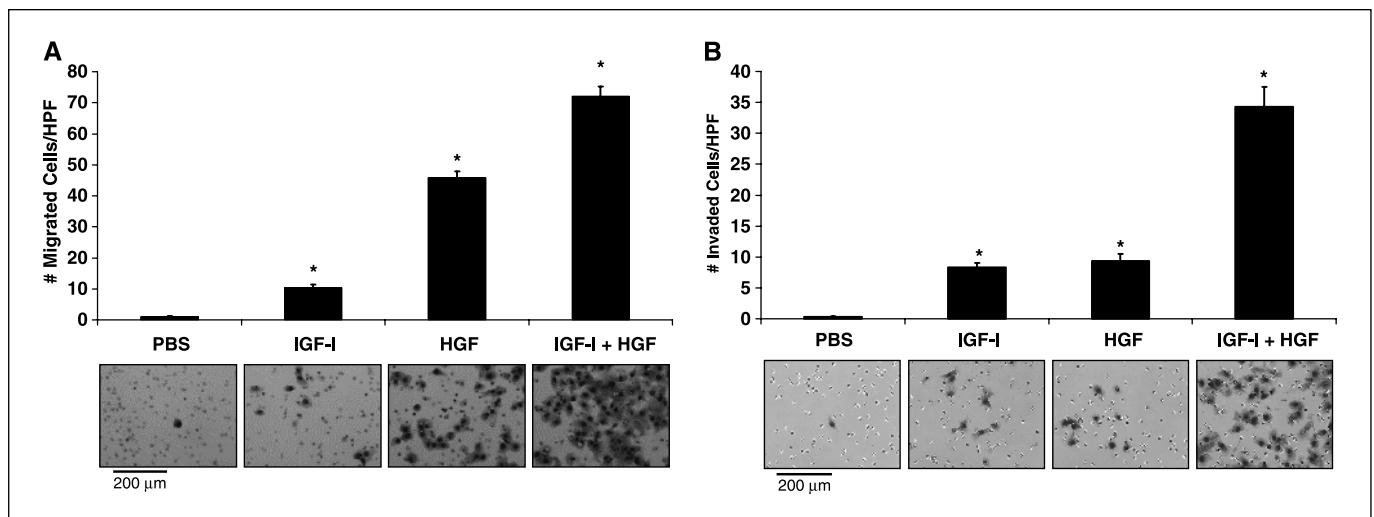
$10 \pm 1$ -fold ( $\pm$ SE) increase in cell migration ( $P < 0.0001$  versus PBS), and HGF led to a mean  $46 \pm 2$ -fold increase in cell migration ( $P < 0.001$  versus PBS; Fig. 1A). The combination of IGF-I and HGF led to a mean  $72 \pm 3$ -fold increase in cell migration ( $P < 0.0001$ ; Fig. 1A), which was significantly greater than the mean increase with IGF-I or HGF alone ( $P < 0.0001$ ).

**Invasion.** The effect of IGF-I and HGF on L3.6pl cell invasion was evaluated using Matrigel-coated transwell invasion assays. IGF-I led to a mean  $28 \pm 2$ -fold increase in cell invasion ( $P < 0.0001$  versus PBS), and HGF led to a mean  $31 \pm 4$ -fold increase in cell invasion ( $P < 0.0001$  versus PBS; Fig. 1B). The combination of IGF-I and HGF led to a mean  $114 \pm 11$ -fold increase in cell invasion ( $P < 0.0001$  versus PBS; Fig. 1B), which was significantly greater than the mean increase with IGF-I alone ( $P < 0.0001$ ).

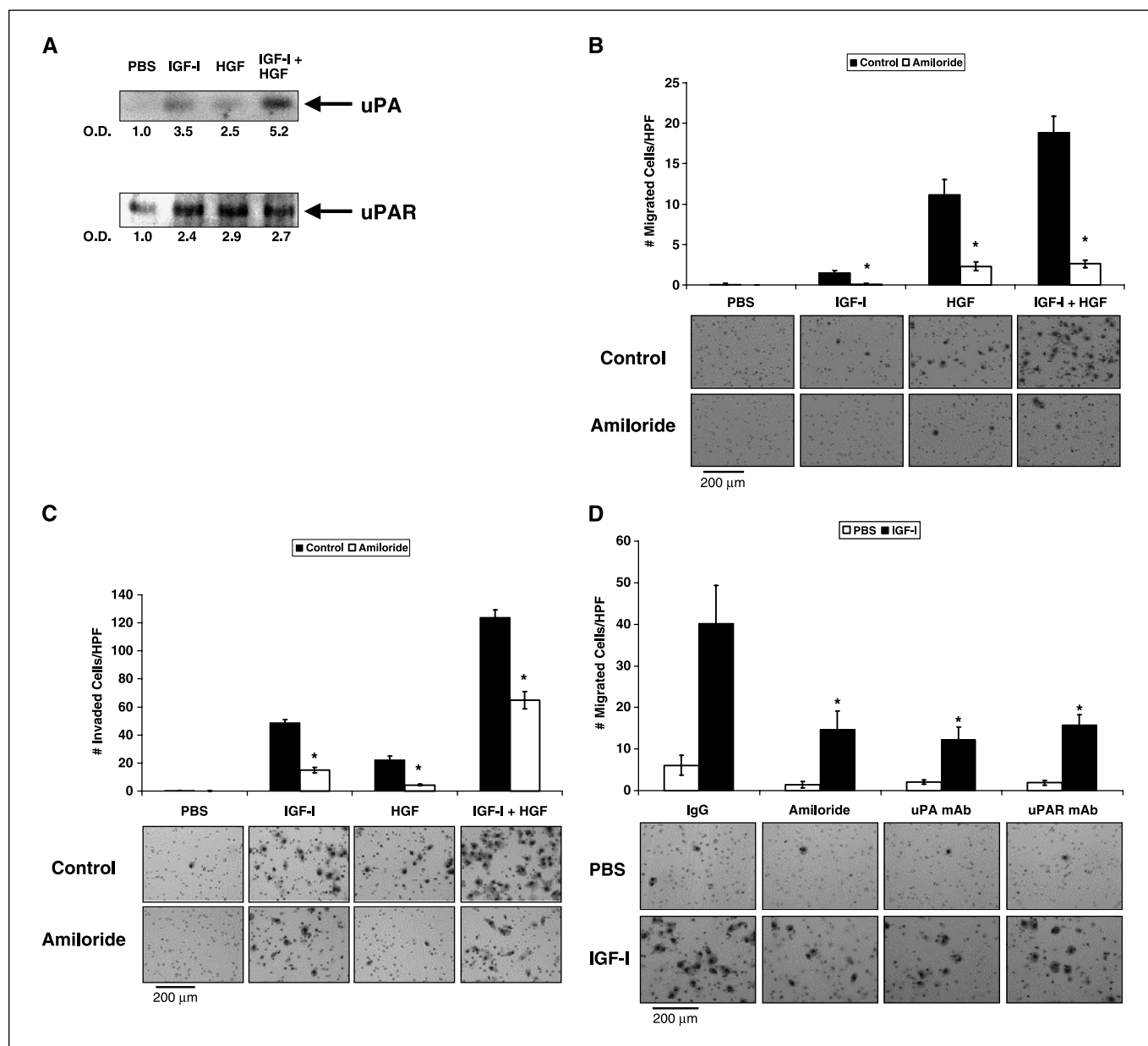
To show that the increases in cell migration and invasion were not due to increases in cell survival induced by IGF-I and HGF, an MTT assay was done on L3.6pl cells treated with PBS, IGF-I, HGF, or IGF-I plus HGF. There was no increase in cytokine-induced cell survival in any of the groups after 24 hours (data not shown).

### Induction of Urokinase-type Plasminogen Activator and Urokinase-type Plasminogen Activator Receptor by Insulin-like Growth Factor-I and Hepatocyte Growth Factor

To evaluate the effect of IGF-I and HGF on the expression of uPA in conditioned media (soluble ligand) and uPAR in cell lysates (cell surface receptor), cells were incubated with PBS, IGF-I, HGF, or IGF-I plus HGF; levels of uPA and uPAR were determined by immunoprecipitation and Western blot analysis. IGF-I led to a 3.5-fold increase in uPA in conditioned media and a 2.4-fold increase in uPAR in cell lysates. (Fig. 2A). HGF led to a 2.5-fold increase in uPA in conditioned media and a 2.9-fold increase in uPAR in cell lysates. The combination of IGF-I and HGF led to a 5.2-fold increase in uPA in conditioned media and a 2.7-fold increase in uPAR in cell lysates. Thus, treatment of cells with IGF-I and HGF increased the secretion of uPA into conditioned media and increased the cell-surface expression of uPAR.



**Figure 1.** Effect of IGF-I and HGF on cell migration and invasion. **A**, a transwell migration assay was done on L3.6pl cells with PBS, IGF-I, HGF, or IGF-I plus HGF. After 24 hours, migrated cells were fixed, stained, and counted. IGF-I and HGF significantly increased cell migration; the effects of IGF-I plus HGF were additive. Representative photographs show migrated cells. \*,  $P < 0.001$  versus PBS; columns, means of 10 high-power fields; bars, SE. **B**, a transwell invasion assay was done on L3.6pl cells with PBS, IGF-I, HGF, or IGF-I plus HGF. After 24 hours, cells were fixed, stained, and counted. IGF-I and HGF increased cell invasion; the effects of IGF-I plus HGF were greater than additive. Representative photographs show migrated cells. \*,  $P < 0.0001$  versus PBS; columns, means of 10 high-power fields; bars, SE.

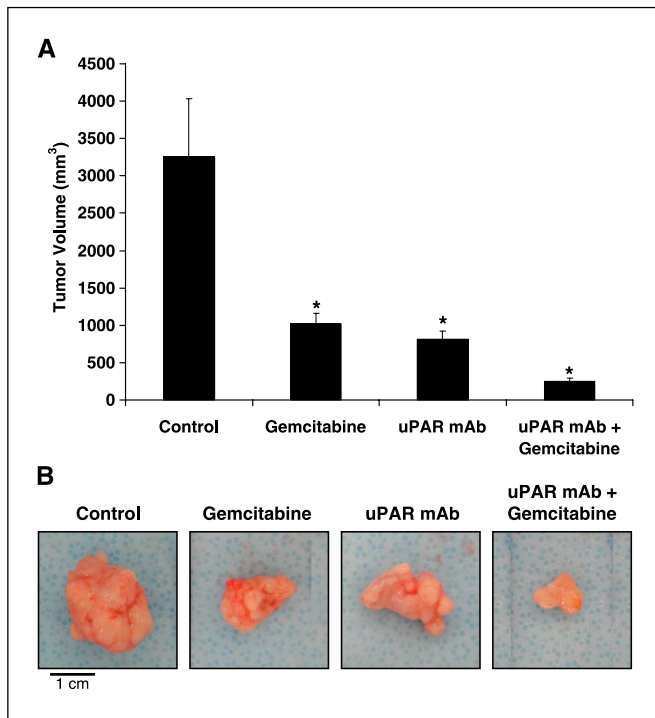


**Figure 2.** Role of uPA and uPAR in cell migration and invasion. **A**, L3.6pl cells were treated with PBS, IGF-I, HGF, or IGF-I plus HGF. Conditioned media and cell lysates were subjected to immunoprecipitation and Western blotting for analysis of uPA and uPAR levels, respectively. IGF-I and HGF led to an increase in uPA and uPAR expression. The combination of IGF-I and HGF led to a further increase in uPA levels above treatment with either cytokine alone but not to a further increase in uPAR levels. *O.D.*, absorbance. **B**, migration assays were done on L3.6pl cells without (control) or with pretreatment with the uPA inhibitor amiloride. uPA inhibition with amiloride decreased cell migration induced by IGF-I, HGF, and IGF-I plus HGF. Representative photographs show migrated cells. \*,  $P < 0.005$  versus control; *columns*, means of 10 high-power fields; *bars*, SE. **C**, invasion assays were done on L3.6pl cells without (control) or with the uPA inhibitor amiloride. uPA inhibition with amiloride led to significant decreases in cell invasion mediated by IGF-I, HGF, and IGF-I plus HGF. Representative photographs show migrated cells. \*,  $P < 0.0001$  versus control; *columns*, means of 10 high-power fields; *bars*, SE. **D**, a transwell migration assay was done on L3.6pl cells. Cells were pretreated with nonspecific IgG, amiloride, anti-uPA mAb, or anti-uPAR mAb, then media was supplemented with PBS (control) or IGF-I. IGF-I induced cell migration in the cells pretreated with IgG. IGF-I-induced cell migration was blocked by amiloride, anti-uPA mAb, and anti-uPAR mAb compared with PBS-treated controls. Representative photographs show migrated cells. \*,  $P < 0.05$  versus IgG; *columns*, means of 10 high-power fields; *bars*, SE.

### Effect of Urokinase-type Plasminogen Activator Inhibition with Amiloride on Cell Migration and Invasion

Given the induction of uPA and uPAR expression by IGF-I and HGF, we assessed whether signaling by the uPA/uPAR system was essential for IGF-I- and HGF-mediated migration and invasion. Migration and invasion assays were repeated with the uPA inhibitor amiloride. Amiloride led to a mean  $93 \pm 7\%$

decrease in IGF-I-mediated migration ( $P < 0.005$ ), a mean  $79 \pm 4\%$  decrease in HGF-mediated migration ( $P < 0.0005$ ), and a mean  $86 \pm 3\%$  decrease in migration with IGF-I plus HGF ( $P < 0.0005$ ; Fig. 2B). In the invasion assays, amiloride led to a mean  $69 \pm 4\%$  decrease in the IGF-I group ( $P < 0.0001$ ), a mean  $81 \pm 3\%$  decrease in the HGF group ( $P < 0.0001$ ), and a mean  $48 \pm 5\%$  decrease in the IGF-I plus HGF group ( $P < 0.0001$ ; Fig. 2C).



**Figure 3.** Effect of anti-uPAR antibody and gemcitabine on pancreatic tumor volume. L3.6pl cells were injected into the pancreatic tails of nude mice. Treatment with nonspecific IgG plus saline (control), gemcitabine, anti-uPAR mAb, or anti-uPAR mAb plus gemcitabine was begun on day 4 and continued until mice were sacrificed on day 28. A, gemcitabine alone inhibited orthotopic tumor growth, as expected. Anti-uPAR mAb alone also significantly inhibited tumor growth, and anti-uPAR mAb plus gemcitabine was better than either agent alone. \*,  $P < 0.05$  versus control; columns, means of 11 to 13 tumors per group; bars, SE. B, representative photographs of pancreatic tumors.

### Effect of Anti-Urokinase-type Plasminogen Activator and Anti-Urokinase-type Plasminogen Activator Receptor Monoclonal Antibodies on Cell Migration

To further investigate the effect of uPA and uPAR inhibition on IGF-I-mediated cell migration, we did migration assays on L3.6pl cells treated with an anti-uPA mAb that inhibits binding of uPA to uPAR and with an anti-uPAR mAb that inhibits downstream signaling by uPAR. Cells were treated with nonspecific IgG for a negative control and amiloride for a positive control. Amiloride resulted in a  $63 \pm 11\%$  decrease in IGF-I-induced cell migration ( $P < 0.05$ ) compared with the IgG control group (Fig. 2D). Pretreatment of the cells with the anti-uPA mAb and anti-uPAR mAb resulted in similar decreases in migration ( $70 \pm 8\%$ ,  $P < 0.01$  versus IgG;  $61 \pm 6\%$ ,  $P < 0.05$  versus IgG, respectively; Fig. 2D).

### Effect of Anti-Urokinase-type Plasminogen Activator Receptor Monoclonal Antibody on Orthotopic Growth, Invasion, and Metastasis of Human Pancreatic Carcinomas in Mice

Because migration and invasion of L3.6pl cells were mediated via a uPAR-dependent pathway, we examined the effect of uPAR inhibition in an *in vivo* model of pancreatic cancer. Mice were treated systemically with gemcitabine and/or the anti-uPAR mAb to assess the effect on orthotopic growth of human pancreatic carcinoma. Gemcitabine treatment led to a mean  $69 \pm 4\%$  smaller pancreatic tumor volume, as expected ( $P < 0.05$  versus control; Fig. 3A and B). Treatment with the anti-uPAR mAb alone led to a mean  $75 \pm 3\%$  smaller pancreatic tumor volume ( $P < 0.01$  versus control). Treatment with gemcitabine plus the anti-uPAR mAb led to a mean  $92 \pm 1\%$  smaller tumor volume ( $P < 0.005$  versus control), which was a significantly greater reduction than with either agent alone ( $P = 0.0002$  versus each).

The primary pancreatic tumors were evaluated for gross evidence of local invasion into the retroperitoneum, and this was confirmed on H&E-stained sections. Gemcitabine alone slightly decreased the incidence of retroperitoneal tumor invasion (67% versus 100% for control,  $P < 0.05$ ). Anti-uPAR mAb, alone and in combination with gemcitabine, completely prevented retroperitoneal invasion by the pancreatic tumors (0% versus 100%,  $P < 0.0001$ ; Table 1 and Fig. 4).

The livers were excised and evaluated for the presence of metastases. Gemcitabine inhibited metastasis to the liver compared with control mice (8% versus 62%,  $P < 0.05$ ; Table 1). Similarly, anti-uPAR mAb alone inhibited liver metastasis (9% versus 62%,  $P < 0.05$ ), as did anti-uPAR mAb plus gemcitabine (8% versus 62%,  $P < 0.05$ ).

### Effect of Urokinase-type Plasminogen Activator Receptor Inhibition on Tumor Cell Proliferation, Tumor Necrosis, Apoptosis, and Angiogenesis

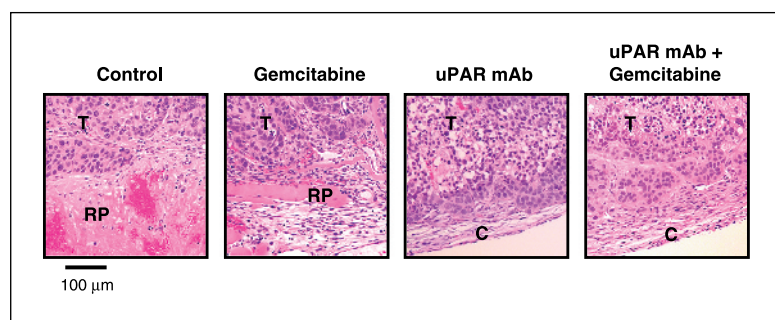
The pancreatic tumors were stained with H&E, anti-activated caspase-3 antibodies, and anti-CD31 antibodies to evaluate the effect of anti-uPAR mAb therapy on necrosis, apoptosis, and angiogenesis, respectively. There were no discernable differences in the percent area of tumor necrosis, apoptosis, or angiogenesis in the anti-uPAR mAb-treated groups compared with control group. To evaluate the effect of anti-uPAR mAb and/or gemcitabine treatment on tumor cell proliferation, mice were injected i.p. with BrdUrd 16 hours before harvest of the tumors. Tumor sections were stained with anti-BrdUrd antibodies, and the number of BrdUrd-positive cells per high-power field was determined. Gemcitabine alone had no effect on tumor cell proliferation (Fig. 5A and B). Anti-uPAR mAb alone led to a mean  $72 \pm 4\%$  reduction in tumor cell proliferation ( $P < 0.0001$

**Table 1.** Effect of uPAR mAb and gemcitabine on retroperitoneal tumor invasion and liver metastasis

	Control	Gemcitabine	uPAR mAb	uPAR mAb + gemcitabine
Retroperitoneal invasion	13 of 13 (100%)	8 of 12 (67%)*	0 of 11 (0%) <sup>†</sup>	0 of 13 (0%) <sup>†</sup>
Liver metastasis	8 of 13 (62%)	1 of 12 (8%)*	1 of 11 (9%)*	1 of 13 (8%)*

\* $P < 0.05$  versus control.

<sup>†</sup> $P < 0.0001$  versus control.



**Figure 4.** Effect of anti-uPAR antibody and gemcitabine on retroperitoneal tumor invasion. Photographs demonstrating retroperitoneal invasion by tumors treated with nonspecific IgG plus saline (control), gemcitabine, anti-uPAR mAb, or anti-uPAR mAb plus gemcitabine. Pancreatic tumor cells invaded the retroperitoneum in the control and gemcitabine-alone groups, whereas there was no retroperitoneal invasion and tumor capsules remained intact in the groups treated with anti-uPAR mAb and anti-uPAR mAb plus gemcitabine. *T*, tumor; *RP*, retroperitoneum; *C*, tumor capsule.

versus IgG control; Fig. 5*A* and *B*). Anti-uPAR mAb plus gemcitabine led to a mean  $85 \pm 2\%$  reduction in proliferation ( $P < 0.0001$  versus control), which was a greater reduction than with anti-uPAR mAb alone ( $P < 0.01$ ).

## Discussion

Tumor cell migration and invasion are critical steps in tumor progression and metastasis, and the uPA/uPAR system has been shown to mediate these processes. Pancreatic carcinomas have been shown to overexpress uPA and uPAR (5, 42), and higher tumor levels of uPAR have been correlated with poor prognosis in patients with pancreatic cancer (27, 28). We hypothesized that (*a*) IGF-I and HGF induce pancreatic carcinoma cell migration and invasion via uPA/uPAR activities and (*b*) uPAR inhibition decreases primary tumor growth, retroperitoneal invasion, and liver metastasis of human pancreatic carcinomas grown orthotopically in mice.

In this study, we found that IGF-I and HGF increased migration and invasion of the human pancreatic carcinoma cell line L3.6pl.

Additionally, IGF-I and HGF led to induction of uPA and uPAR in L3.6pl cells. Inhibition of IGF-I- and HGF-mediated migration and invasion by the uPA inhibitor amiloride or by neutralizing antibodies to uPA and uPAR indicated that uPA and uPAR are critical for optimal tumor cell migration and invasion. This link between IGF-I signaling and uPA/uPAR activation has not been previously shown in pancreatic cancer.

Inhibition of uPAR in orthotopic pancreatic tumors via systemic mAb treatment in mice showed that uPAR function is important in primary tumor growth, retroperitoneal invasion, and liver metastasis of human pancreatic carcinoma. Immunohistochemical staining of the pancreatic tumors with anti-BrdUrd antibodies showed that uPAR inhibition led to a decrease in tumor cell proliferation. uPAR has been shown to mediate signaling intermediates that control the mitogenic pathway (31); thus, these findings, although novel in pancreatic cancer, are consistent with what is known about uPAR signaling.

uPAR has been implicated in angiogenesis because of its role in endothelial cell invasion (43, 44). In our study, we did not note a decrease in vessel count in orthotopic tumors, but this may have been due to the specificity of the anti-uPAR mAb. The anti-uPAR mAb used in our studies does not recognize murine uPAR, and thus the antibody would not have a direct effect on murine endothelial cells.

Local tumor invasion is of great significance in pancreatic carcinoma because it precludes potentially curative surgical resection in ~40% of patients. Additionally, the presence of tumor cells at the surgical resection margin is an independent predictor of shorter survival in patients with pancreatic carcinoma (45, 46). Our finding that uPAR inhibition led to complete inhibition of retroperitoneal invasion by primary pancreatic tumors in mice may, therefore, have therapeutic implications.

In conclusion, our study shows the importance of the uPA/uPAR system in pancreatic carcinoma cell migration and invasion and suggests that uPAR is a potential target for therapy.

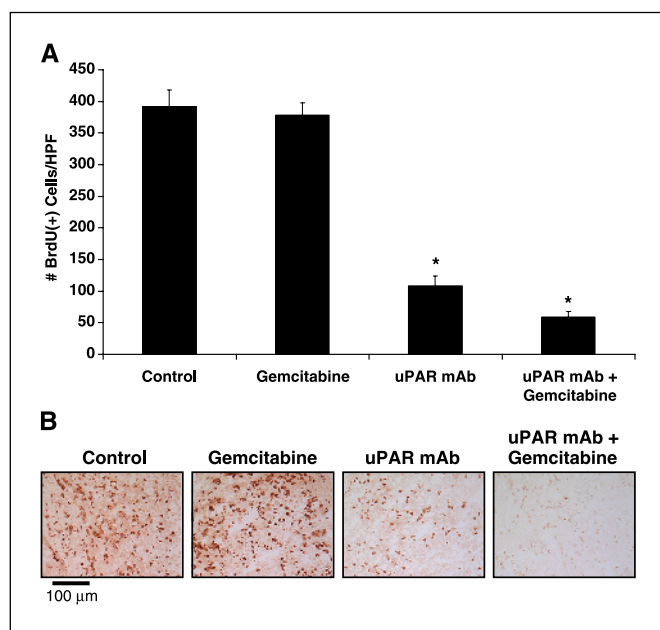
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**Figure 5.** Effect of anti-uPAR antibody and gemcitabine on tumor cell proliferation. Mice were injected with BrdUrd before tumor harvest. Tumor sections were stained with anti-BrdUrd antibodies. *A*, anti-uPAR mAb significantly inhibited tumor cell proliferation, and this effect was enhanced with the addition of gemcitabine. \*,  $P < 0.0001$  versus control; *columns*, means of 11 to 13 tumors per group; *bars*, SE. *B*, representative photographs of anti-BrdUrd-stained tumors sections.

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## Targeting of Urokinase Plasminogen Activator Receptor in Human Pancreatic Carcinoma Cells Inhibits c-Met– and Insulin-like Growth Factor-I Receptor–Mediated Migration and Invasion and Orthotopic Tumor Growth in Mice

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