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.

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...
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 invasation (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 L.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% CO2/95% air at 37°C. Growth curves 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 (37). To determine the effect of IGF-I and HGF on Western blot analysis for determination of uPA protein expression, as previously described (38).

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, or IGF-I plus HGF (100 ng/mL each). Assays were done in triplicate to achieve a final concentration of 100 ng/mL 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. The uPA inhibitor amiloride (100 μmol/L; Sigma-Aldrich Co., St. Louis, MO) was added (50 μmol/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.

Orthotopic tumor model. L3.6pl cells were grown to 80% confluency and trypsinized, and cell viability was confirmed by trypan blue exclusion. Cells (1 × 106 cells/mouse) were injected into the tail of the pancreas 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: (a) IGF-I plus saline, (b) IGF-I plus gemcitabine, (c) anti-uPA mAb plus saline, and (d) anti-uPAR mAb plus gemcitabine. Gemicitabine (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).

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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 length × (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 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 ± 1-fold (±SE) increase in cell migration (P < 0.0001 versus PBS), and HGF led to a mean 46 ± 2-fold increase in cell migration (P < 0.0001 versus PBS; Fig. 1A). The combination of IGF-I and HGF led to a mean 72 ± 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 ± 2-fold increase in cell invasion (P < 0.0001 versus PBS), and HGF led to a mean 31 ± 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 ± 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 uPA 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 uPA 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 uPA 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](https://example.com/figure1.png)
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 ± 7% decrease in IGF-I–mediated migration (P < 0.005), a mean 79 ± 4% decrease in HGF-mediated migration (P < 0.0005), and a mean 86 ± 3% decrease in migration with IGF-I plus HGF (P < 0.0005; Fig. 2B). In the invasion assays, amiloride led to a mean 69 ± 4% decrease in the IGF-I group (P < 0.0001), a mean 81 ± 3% decrease in the HGF group (P < 0.0001), and a mean 48 ± 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; †, P < 0.0001 versus control. B, representative photographs of pancreatic tumors.

Effect of 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 ± 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 ± 8%, P < 0.01 versus IgG; 61 ± 6%, P < 0.05 versus IgG, respectively; Fig. 2D).

Effect of Anti–Urokinase-type Plasminogen Activator Receptor Monoclonal Antibody on Orthotopic Pancreatic Tumor 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 ± 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 ± 3% smaller pancreatic tumor volume (P < 0.01 versus control). Treatment with gemcitabine plus the anti-uPAR mAb led to a mean 92 ± 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 ± 4% reduction in tumor cell proliferation (P < 0.0001

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

<table>
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<th>Control</th>
<th>Gemcitabine</th>
<th>uPAR mAb</th>
<th>uPAR mAb + gemcitabine</th>
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<tbody>
<tr>
<td>Retroperitoneal invasion</td>
<td>13 of 13 (100%)</td>
<td>8 of 12 (67%)*</td>
<td>0 of 11 (0%)†</td>
<td>0 of 13 (0%)‡</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>8 of 13 (62%)</td>
<td>1 of 12 (8%)*</td>
<td>1 of 11 (9%)*</td>
<td>1 of 13 (8%)*</td>
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*P < 0.05 versus control.
†P < 0.0001 versus control.
versus IgG control; Fig. 5A and B). Anti-uPAR mAb plus gemcitabine led to a mean 85 ± 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.

Acknowledgments

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