Suppression of the uPAR–uPA System Retards Angiogenesis, Invasion, and In Vivo Tumor Development in Pancreatic Cancer Cells

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Abstract

Despite existing chemotherapy and surgical resection strategies, pancreatic cancer is one of the major causes of mortality in the United States with a 5-year mean survival rate of less than 5%. The activation of the urokinase-type plasminogen activator receptor–urokinase-type plasminogen activator (uPAR–uPA) system in the development of pancreatic ductal adenocarcinoma has been well established. In the present study, we used 2 pancreatic cancer cell lines, MIA PaCa-2 and PANC-1 to show the effects of uPAR and uPA downregulation. From the results, we observed that RNAi expressing plasmids efficiently downregulated mRNA and protein expression of uPAR and uPA. In vitro and in vivo angiogenic assays revealed a significant decrease in the angiogenic potential of MIA PaCa-2 and PANC-1 cells that were downregulated for both uPAR and uPA. From the angiogenesis antibody array analysis, we observed that the simultaneous downregulation of uPAR and uPA resulted in the downregulation of angiogenin and overexpression of RANTES. Further, FACS analysis showed that the simultaneous downregulation of uPAR and uPA caused the accumulation of cells in the sub-G0/1 phase in both MIA PaCa-2 and PANC-1 cells. In addition, Western blot analysis revealed that downregulation of uPAR and uPA caused the activation of caspase 8 and CAD, which is indicative of apoptosis, and in vivo TUNEL assay confirmed these results. Finally, we observed the nuclear localization of uPA and that uPA interacts with the transcription factor Lhx-2. Taken together, the results of the present study show that the targeting of the uPAR–uPA system has therapeutic potential.

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Introduction

Each year about 37,000 individuals in the United States are diagnosed with pancreatic cancer, and more than 30,000 die from the disease. Depending on the size of the tumor at the time of diagnosis, prognosis is generally poor with less than 5% still alive 5 years after diagnosis, and complete remission is extremely rare (1). Treatment of pancreatic cancer depends on the stage of the cancer. Gemcitabine was approved by the Food and Drug Administration (FDA) in 1998 after a clinical trial reported improvements in quality of life in patients with advanced pancreatic cancer; this marked the first FDA approval of a chemotherapy drug for a nonsurvival clinical trial endpoint (2). Patients diagnosed with pancreatic cancer typically have a poor prognosis partly because the cancer usually does not cause symptoms at earlier stages, which leads to locally advanced or metastatic disease by the time of diagnosis. The median survival rate is 3 to 6 months after diagnosis and, as mentioned previously, the 5-year survival rate is less than 5% (3). Although it accounts for only 2.5 percent of new cases, pancreatic cancer is responsible for 6% of cancer deaths each year (4). The survival rate (3) and high recurrence after surgery clearly show the need for novel therapies (5).

Importance of the uPAR–uPA system in pancreatic cancers

Recent studies have suggested that the expression levels of urokinase-type plasminogen activator (uPA) and fibroblast-type uPA receptor (uPAR) are correlated with liver metastasis of human pancreatic cancer (6), indicating the relevance of specifically targeting the uPAR–uPA system. Other studies have showed similar results and have shown the importance of the uPAR–uPA system in pancreatic carcinoma cell migration (7). Invasion studies also suggest that uPAR is a potential target for therapy in patients with pancreatic cancer (1, 6–10). In addition, studies have shown the influence of the insulin-like growth factor (IGF) system on tumor progression from benign mucinous cystadenomas to pancreatic ductal adenocarcinoma (PDAC; ref. 11).
However, the uPAR–uPA system had a greater influence on survival of PDAC (11), which highlights the importance of targeting the uPAR–uPA system.

**uPAR–uPA associated complex**

Malignant tumors have the capacity to degrade the extracellular matrix (ECM) by controlled proteolysis. One proteolytic system involved in these processes is the uPA system, which consists of uPA, the uPAR, and uPA inhibitors 1 and 2, plasminogen activator inhibitor (PAI) 1 and 2. A growing body of evidence suggests that the uPA system promotes tumor metastasis by several different mechanisms and not solely through the breakdown of the ECM (12). Initially, uPAR was thought to function simply as a mechanism to concentrate the urokinase/plasmin system near the cell surface. However, extensive evidence has since shown that this glycolipid-anchored receptor also functions in both the adhesive and signaling pathways of many migratory cells. One mechanism through which uPAR directs these actions is bycomplexing with other membrane proteins (e.g., integrins) for signal transduction. There are 3 basic steps involved in invasion and intracellular signaling: (1) uPAR–uPA promotes extracellular proteolysis by regulating plasminogen activation, (2) uPAR–uPA regulates cell/ECM interactions as an adhesion receptor for vitronectin (Vn) and through its capacity to modulate integrin function, and (3) uPAR–uPA regulates cell migration as a signal transduction molecule and by its intrinsic chemotactic activity (13).

Recent studies point to important structural features of uPAR-integrin interactions, suggesting uPAR as a cis-acting integrin ligand (14). Although uPAR transcripts are induced by adhesion, rapid synthesis of the protein uses constitutive mRNA without a requirement for new transcription and is regulated by mammalian target of rapamycin, demonstrating new biologic roles for the signal-dependent translation pathway controlled by this intracellular kinase (15). Integrins are known to activate PI3K and MEK pathway, and higher activation of Akt is associated with increased phosphorylation of glycogen synthase kinase 3β (GSK3β), FKHR, and mTOR (16), indicating a possible feedback mechanism. Researchers have shown that urokinase-induced signaling in human vascular smooth muscle cells is mediated by platelet derived growth factor receptor (PDGFR)-β (17), which shows its multifaceted role in signaling. In the present study, we further delineated the complex role of uPAR and its associated molecules in activating apoptosis in pancreatic cancer.

**Materials and Methods**

**Cell lines and culture conditions**

MIA PaCa-2 and PANC-1 cells were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS in a humidified 5.0% CO₂ atmosphere at 37.2°C with media changes every 48 hours.

**Tissue array**

Human pancreatic cancer tissue arrays were obtained from US Biomax, Inc. The tissue arrays consisted of pancreatic cancer and normal tissues with stage and grade information of 101 cases, consisting of 208 cores (PA2082-024 and -025). Hematoxylin and eosin (H&E)-stained core images are available from the manufacturer (http://www.biomax.us/tissue-arrays/Pancreas/PA2082). Tissue arrays were processed for immunohistochemistry as per standard protocol. Briefly, slides were deparaffinized and endogenous peroxidases activity was blocked at room temperature by 5 to 10 minutes incubation in 0.3% H₂O₂ in PBS (pH 7.7). The slides were then rinsed in PBS for 5 minutes followed by antigen retrieval by heating to about 95°C in citrate buffer (0.01 mol/L sodium citrate buffer, pH 6.0) for 10 minutes. The tissues were blocked using normal goat serum for 20 minutes at room temperature and followed by addition of primary antibody (anti-uPA or anti-uPAR) for 60 minutes at room temperature. The slides were then rinsed in PBS followed by the addition of a horseradish peroxidase (HRP)-conjugated secondary antibody for 20 minutes at room temperature, HRP substrate DAB (3,3’-diaminobenzidine tetrahydrochloride) at 1 mg/mL in 50 mmol/L Tris, pH 7.2, and 0.3% H₂O₂ was then added for the development of DAB substrate followed by H&E staining. Next, the slides were dehydrated and mounted with cover slips. Transmitted light images were obtained after hematoxylin staining to determine uPAR and uPA expression levels.

**Construction of short hairpin RNA-expressing plasmid**

Short hairpin RNA (shRNA)-expressing plasmids were constructed as described previously (18, 19).

**Reverse transcription-PCR analysis**

MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPA, puPAR, or pU2 for 72 hours. The cells were collected and total cell RNA was isolated. Reverse transcriptase PCR was set up using primers specific for uPAR and uPA (Table 1). The PCR cycle was: 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, repeated 30 times. The product was quantified and plotted relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as arbitrary units.

**Antibodies**

The following antibodies were obtained from Santa Cruz Biotechnology: anti-uPA (sc-14019), anti-uPAR (sc-13522), anti-Lhx-2 (sc-81311), anti-GAPDH (sc-59541), anti-CAD (caspase-activated Dnase; sc-8342), and anti-Lamin-B (sc-6216). We purchased anticleaved caspase 8 (9496) from Cell Signaling Technology.

**Western blotting**

MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPA, puPAR, or pU2 for 72 hours. Then, cells were collected, and total cell lysates were prepared in standard...
radioimmunoprecipitation assay buffer (RIPA) extraction buffer containing apotinin and phenylmethylsulfonylfluoride. Twenty micrograms of protein from these samples were separated under nonreducing conditions by 12% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were immunoprobed for uPAR, cleaved caspase 8, CAD, and GAPDH as per standard protocols. The membranes were treated with the appropriate HRP-conjugated secondary antibody and then developed according to enhanced chemiluminescence protocol (Amersham). Similarly, nuclear extracts were immunoprobed for uPA and Lhx-2; for controls, membranes were stripped and probed with monoclonal antibodies for Lamin B as per standard protocol.

Fibrin zymography

The enzymatic activity and molecular weight of electrophoretically separated forms of uPA in the conditioned media of MIA PaCa-2 and PANC-1 cells transfected with pSV, puPAR, puPA, or pU2 were determined by SDS-PAGE as described previously (20, 21). As stated, the acrylamide gels were enriched with purified plasminogen and fibrinogen before polymerization. Equal amounts of sample proteins were electrophoresed, and the gels were washed and stained to determine enzymatic activity as per standard protocol.

In vitro angiogenic assay

MIA PaCa-2 and PANC-1 cells (2 × 10⁶/well) were seeded and transfected with pSV, puPAR, puPA, or pU2. After a 24-hour incubation period, the conditioned medium was removed and added to a 4 × 10⁵ human dermal endothelial cell in 8-well chamber slides. Cells were allowed to grow for 72 hours. Cells were then fixed in 3.7% formaldehyde and H&E stained to visualize capillary network formation. The degree of angiogenesis was quantified based on the numerical value for the product of the number of branches and number of branch points as an average of 10 fields. The results are graphically represented.

In vivo angiogenic assay

The implantation technique of the dorsal skin-fold chamber model has been described previously (22). Sterile small animal surgical techniques were followed. Diffusion chambers were filled with 2 × 10⁶ MIA PaCa-2 and PANC-1 cells, which were transfected with pSV, puPAR, puPA, or pU2 and suspended in 100 to 150 μL of sterile PBS. Mice were anesthetized using isoflurane [2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane]-saturated oxygen gas. Once the animal was anesthetized completely, a dorsal air sac was made in the mouse by injecting 10 mL of air. A 1.5 to 2 cm superficial incision was made horizontally along the edge of the dorsal air sac, and the air sac was opened. With the help of forceps, the chambers were placed underneath the skin and sutured carefully. After 10 days, the animals were anesthetized with ketamine/xylazine and sacrificed by intracardiac perfusion with saline (10 mL) followed by a 10 mL of 10% formalin in 0.1 mol/L standard phosphate buffer. The animals were carefully skinned around the implanted chambers and the implanted chambers were removed from the air sac fascia. The skin-fold covering the chambers was photographed under visible light. The number of blood vessels within the chamber in the area of the air sac fascia was counted and their lengths measured.

Angiogenesis antibody array

Angiogenic antibody array was performed as per the manufacturer’s instructions (Ray Bio Human Angiogenesis Antibody Array C Series). Briefly, MIA PaCa-2 and PANC-1 cells (2 × 10⁶/well) were seeded and transfected with pU2. After a 24-hour incubation period, the conditioned medium was removed and membranes were incubated with conditioned media. Following incubation, the membranes were washed and 1 mL of diluted biotin-conjugated antibody mix was added to the membranes and incubated at room temperature for 1 to 2 hours, followed by washing and addition of diluted HRP-conjugated streptavidin. Detection was based on ECL-protocols where membranes were exposed to X-ray films to visualize binding of angiogenic factors to membranes, which are seen as spots of varying intensities. Positive controls were used to normalize the results from different membranes being compared. Spot intensities were quantified using Image J software, and angiogenic molecules showing significant change in expression levels were identified.

Matrigel invasion assay

The in vitro invasiveness of MIA PaCa-2 and PANC-1 cells in the presence of the vector expressing siRNA for uPAR and uPA was assessed using a modified Boyden chamber assay. MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPAR, puPA, or pU2 for 48 hours. 1 × 10⁶ cells were suspended in 200 μL of serum-free medium supplemented with 0.2% bovine serum albumin (BSA).
and placed in the upper compartment of transwell chambers (Corning Costar Fischer Scientific Catalogue no. 07-200-158) coated with Matrigel (0.7 mg/mL). The lower compartment of the chamber was filled with 700 µL of serum-free medium, and the cells were allowed to migrate for 24 hours. After incubation, the cells that passed through to the lower chamber were fixed and stained with Hema-3 and quantified as previously described (23).

**Cell cycle analysis**

MIA PaCa-2 and PANC-1 cells (2 × 10⁶) were transfected with pSV, puPAR, puPA, or pU2 for 48 hours. Cells were then trypsinized and treated with 50 µg/mL propidium iodide + 0.001% RNase A solution as per standard protocol. The cells were sorted on a fluorescence-activated cell sorter and quantified (10,000 cells sorted per treatment condition with 3 replications).

**Orthotopic tumor model**

Subcutaneous tumor growth. We carried out s.c. implantation as previously described (24). Briefly, MIA-PaCa-2 cells were harvested by trypsinization and washed with PBS. Cells were injected s.c. into mice in a total volume of 0.2 mL within 30 minutes of harvesting. The s.c. tumors were used as the source of orthotopic implantation of tissue onto the pancreas.

Surgical orthotopic implantation of MIA-PaCa-2 tumors. The following procedure was performed as described by Katz and colleagues (25). MIA-PaCa-2 tumors in the exponential growth phase, grown s.c. in nude mice, were resected aseptically. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into 1 mm³ pieces in DMEM. Mice were then anesthetized using isoflurane (2-chloro-2-difluoromethoxy)-1, 1, 1-trifluoro-ethane)-saturated oxygen gas, and their abdomens were sterilized with alcohol. An incision was created through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed, and 2 tumor pieces were transplanted onto the middle of the gland using a single surgical suture. The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed in 2 layers using 6-0 surgical suture. Three days after implantation, mice were given i.p. injections of pU2 5 times at 150 µg per mouse every other day. Thirty days after implantation, mice were sacrificed and pancreatic tissues were isolated and processed for paraffin embedding.

**Immunohistochemical analysis**

Pancreatic tissues with tumors of control (untreated), pSV- and pU2-treated mice implanted with MIA PaCa-2 tumors were fixed in formaldehyde and embedded in paraffin as per standard protocols. Sections were deparaffinized as per standard protocol, pretreated with 0.3% H₂O₂ to inactivate native peroxidases followed by incubation in 1% BSA in PBS for 1 hour, and subsequently transferred to primary antibody (uPAR, uPA, or Lhx-2) diluted in 1% BSA in PBS (1:500). Sections were allowed to incubate in the primary antibody solution for 2 hours at room temperature in a humidified chamber, followed by washes (3 at 10 min each) in 1% BSA in PBS and placed in a solution with the appropriate HRP-conjugated secondary antibody. The sections were allowed to incubate with the secondary antibody for 1 hour followed by washes in PBS (5 for 5 min each). HRP substrate DAB at 1 mg/mL in 50 mmol/L Tris (pH 7.2) and 0.3% H₂O₂ was then added for the development of DAB substrate. Transmitted light images were obtained after hematoxylin staining as per standard protocol to visualize the morphology of the sections. A control study was performed using a normal rabbit immunoglobulin fraction as the primary antibody (control Ab) instead of uPAR, uPA, or Lhx-2.

**In situ TUNEL assay**

A terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) apoptosis detection kit (Upstate Biotechnology Inc.) was used for DNA fragmentation fluorescence staining and carried out according to the manufacturer’s protocol. Briefly, MIA PaCa-2 tumor sections from control, pSV- and pU2-treated mice were incubated with a reaction mix containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 minutes. Fluorescein-conjugated avidin was applied to the sample, which was then incubated in the dark for 30 minutes. Positively stained fluorescein-labeled cells were visualized after 4’,6-diamidino 2 phenylindole (DAPI) staining using fluorescence microscopy and quantified as percent positive cells relative to cell density.

**Immunofluorescence**

MIA PaCa-2 and PANC-1 cells grown on chamber slides were transfected with pSV, puPAR, puPA, or pU2. After 72 hours, cells were fixed with 3.7% formaldehyde and incubated with 1% BSA in PBS at room temperature for 1 hour for blocking. Then, the slides were washed with PBS and incubated with IgG anti-uPA (rabbit) and IgG anti-Lhx-2 (mouse) at a concentration of 1:200. The slides were incubated at room temperature for 4 hours and washed 3 times with PBS to remove excess primary antibody. Cells were then incubated with anti-rabbit FITC-conjugated IgG (1:50 dilution) and anti-mouse Texas-Red conjugated IgG (1:50 dilution). Next, the slides were washed 3 times, covered with glass cover slips using a DAPI containing mounting media and fluorescent photomicrographs were obtained using a triple filter cube red, green, or blue (RGB).

**Transcription factor binding array analysis**

Transcription Factor (TF) protein–protein binding array was done using the TF Protein Array kit from Panomics (Catalogue no. MA3501-08) as per the manufacturer’s instructions. Briefly, human uPA (huPA) protein (American Diagnostics) was suspended in 1X blocking buffer at a concentration of 100 ng/mL. The TF membranes were incubated in 1X blocking buffer for 2 hours at room temperature (RT) followed by incubation with huPA in
1X blocking buffer for 2 hours at RT. This was followed by washings and further incubation with anti-uPA antibody followed by secondary HRP-conjugated antibody as per kit instructions. Detection for HRP was done using the provided detection buffers followed by exposure of membranes to X-ray film. Binding of huPA to TFs was observed as spots on the X-ray film.

Statistical analysis
All experiments were repeated in triplicate with the exception of the human pancreatic tissue array. A P value of 0.01 or less was considered significant.

Results

Human pancreatic carcinoma and adenocarcinoma show increased expression of uPAR and uPA
We analyzed human pancreatic tissue arrays to determine expression levels of both uPAR and uPA by immunohistochemistry. Identical tissue arrays obtained by serial sectioning were used for this study. We observed that, expression of both uPAR and uPA was colocalized in most cases (Fig. 1A). uPA expression was seen uniformly throughout the tumor tissue with little expression in the large excretory ducts, whereas uPAR expression was observed in the tumor tissues as well as in the excretory ducts (Fig. 1B and C). In most tissues, overexpression of uPA was accompanied by overexpression of uPAR. Expression of uPAR and uPA with grade information is provided in the supplementary data.

Plasmids expressing shRNA targeting uPAR and uPA suppress uPAR and uPA mRNA and protein levels in MIA PaCa-2 and PANC-1 cells
MIA PaCa-2 and PANC-1 cells were transfected with plasmids expressing shRNA targeting uPAR (puPAR), uPA (puPA), and uPAR–uPA simultaneously (pU2). Cell lysates were used to determine uPAR expression levels and the conditioned media was used to determine uPA activity by fibrin zymography. Figure 2A shows a 2- and 4-fold decrease in the mRNA levels of uPAR in puPAR- and puU2-transfected cells when compared to pSV-transfected and control cells, respectively. Similarly, we observed a significant (>2-fold) decrease in the mRNA levels of uPA in puPA- and pU2-transfected cells as compared to pSV-transfected and control cells (Fig. 2A). From the fibrin zymography results, we observed a decrease in uPA activity in puPA and pU2-transfected cells as compared...

Figure 1. Human pancreatic cancer tissues show overexpression of both uPAR and uPA. Tissue arrays consisted of pancreatic cancer and normal tissues with stage and grade information of 101 cases consisting of 208 cores (PA2082-024 and -025). Tissue arrays were processed for immunohistochemistry as per standard protocols. A, expression of uPAR and uPA was observed as brown coloration of DAB reaction. B, microscopic examination at 4X and 10X magnifications was done to observe tumor tissue morphology. C, microscopic examination of the large excretory ducts was done to determine invasion of tumor tissue.
to pSV-transfected and control cells by more than 2- and 4-fold in both cell lines, respectively (Fig. 2B and C). uPAR protein expression decreased by 3-fold in puPAR-transfected cells and 4-fold in pU2-transfected cells as compared to the controls (Fig. 2B–D). Cells transfected with puPA had decreased uPA activity but no significant change in uPAR expression levels (Fig. 2B–D).

Suppression of uPAR and uPA expression in MIA PaCa-2 and PANC-1 cells retards their invasive potential, causes the accumulation of cells in the sub-Go1 phase, and activates caspase-8-mediated apoptosis.

To study the effect of pU2 on pancreatic cancer cell invasion, we used the Matrigel invasion model. MIA PaCa-2 and PANC-1 cells were transfected with puPAR, puPA, or...
pU2 and were allowed to invade through Matrigel for 24 hours. No significant difference was observed in control and pSV-transfected cells, while decreased invasion was observed in cells transfected with puPAR, puPA, or pU2 (Fig. 3A). Quantitative analysis revealed that invasion decreased by 20% \pm 3% in MIA PaCa-2 cells and by 47% \pm 1% in PANC-1 cells after puPAR transfection. Cells transfected with puPA showed similar decreases in invasion (MIA PaCa-2: 18% \pm 2% and PANC-1: 45% \pm 4%). Cells transfected with pU2 showed the maximum decrease in invasion (MIA PaCa-2: 57% \pm 2% and PANC-1: 63% \pm 4%) when compared to all other treatment conditions (Fig. 3A). To further determine whether the downregulation of uPA caused changes in cell cycle progression, MIA PaCa-2 and PANC-1 cells were transfected with pSV, puPAR, puPA, or pU2. From the results of the Fluorescence activated cell sorter (FACS) analysis, we observed that simultaneous downregulation of uPAR and uPA caused a higher accumulation of cells in the sub-G0/G1 phase in both MIA PaCa-2 and PANC-1 cells as compared to downregulation of either uPAR or uPA alone. Controls and pSV-transfected cells had very little accumulation of cells in the sub-G0/G1 phase (Fig. 3B). Quantitative analysis indicated that MIA PaCa-2 and PANC-1 cells transfected with puPAR (MIA PaCa-2: 34% \pm 3% and PANC-1: 36% \pm 3%) or puPA (MIA PaCa-2: 28% \pm 32% and PANC-1: 30% \pm 2%) had increased accumulation of cells in the sub-G0/G1 phase. Cell death in pU2-transfected cells (MIA PaCa-2: 57% \pm 2% and PANC-1: 58% \pm 3%) was found to be more than 50% (Fig. 3B). We next sought to...
and PANC-1: 15%.

In contrast, cells transfected with puPAR and puPA had increased caspase 8 cleavage (MIA PaCa-2: 12% ± 2% and PANC-1: 15% ± 3%) and CAD expression (MIA PaCa-2: 6% ± 2% and PANC-1: 9% ± 3%). We observed the most significant increase in CAD expression in pU2-transfected cells (MIA PaCa-2: 65% ± 2% and PANC-1: 72% ± 3%; Fig. 3C).

**Suppression of uPAR and uPA expression in MIA PaCa-2 and PANC-1 cells retards their angiogenic potential in vitro and in vivo**

To assess whether downregulation of uPAR and uPA can influence tumor-induced angiogenesis in an in vitro system, we cultured human endothelial cells with conditioned media from MIA PaCa-2 or PANC-1 cells transfected with pSV, puPAR, puPA, or pU2. Endothelial cells formed dense capillary-like structures when cultured with conditioned media from untransfected or pSV-transfected MIA PaCa-2 or PANC-1 cells. Endothelial cells grown in conditioned media of MIA PaCa-2 or PANC-1 cells transfected with puPAR or puPA showed a decrease in the density and completeness of the capillary network structure as compared to the controls. We observed broken and rudimentary network formation in endothelial cells grown in conditioned media from pU2-transfected MIA PaCa-2 or PANC-1 cells (Fig. 4A). Quantitative analysis indicated that transfection with puPAR (MIA PaCa-2 37% ± 3% and PANC-1: 55% ± 4%) or puPA (MIA PaCa-2: 36% ± 2% and PANC-1: 41% ± 2%) decreased capillary network formation (controls considered as 100% angiogenic). Transfection with pU2 (MIA PaCa-2: 68% ± 2% and PANC-1: 78% ± 4%) caused the most significant decrease in endothelial cell network formation (Fig. 4A).

To further show the effect of uPAR and uPA downregulation on tumor angiogenesis, we used the dorsal skin-fold chamber assay in vivo model system. Implantation of a chamber containing MIA PaCa-2 or PANC-1 cells pretransfected with pSV, puPAR, puPA, or pU2 resulted in the development of microvessels with curved thin structures. MIA PaCa-2 or PANC-1 cells transfected with pU2 showed no new development of vasculature, whereas puPAR- and puPA-transfected cells did result in formation of neovascularization but was less than that of control or pSV-transfected cells where maximum neovascular formation was observed (Fig. 4B). Quantitative analysis of neovascularization from the in vivo angiogenesis assay indicated that controls and pSV-treated cells had similar increases in vasculature, whereas puPAR-treated cells (MIA PaCa-2: 28% ± 6% and PANC-1: 36% ± 3%) and puPA-treated cells (MIA PaCa-2: 37% ± 3% and PANC-1: 15% ± 3%) had decreased angiogenesis. pU2-treated cells (MIA PaCa-2: 88% ± 3% and PANC-1: 85% ± 3%) exhibited little increase in vasculature (Fig. 4B).

**Simultaneous downregulation of uPAR and uPA in MIA PaCa-2 and PANC-1 cells regulates expression of angiogenic factors**

To further analyze the effect of pU2 on angiogenic inhibition, we used an antibody array specific for angiogenic factors. We used conditioned media from untransfected and pU2-transfected MIA PaCa-2 and PANC-1 cells. We observed that the expression level of angiogenin reduced significantly (2- to 3-fold decrease) in both MIA PaCa-2 and PANC-1 cells. Tissue inhibitor of metalloproteinase (TIMP)-2 expression levels also decreased 1 to 2-fold. MCP-1 expression level decreased by 4-fold in PANC-1 cells but not in MIA PaCa-2 cells. Similarly, a 2-fold decrease in ENA-78 expression levels was observed in MIA PaCa-2 cells but not in PANC-1 cells. Expression levels of the angiogenic inhibitor RANTEs increased 2- to 3-fold in both cell lines (Fig. 4C).

**Intraperitoneal injections of plasmids expressing shRNA targeting uPAR and uPA induce cell death in MIA PaCa-2 orthotopic tumors**

To determine the in vivo effect of pU2 on pancreatic cancer cells, we used an orthotopic tumor model. Untreated mice pancreas showed normal pancreatic morphology with well-defined acini and islets of Langerhans. In the control and pSV-treated mice, pancreatic tissues were infiltrated with tumor cells. However, in pU2-treated mice, we observed necrotic tumor tissue and decreased tumor cell density when compared to controls. Immunohistochemical analysis for uPAR and uPA expression indicated that normal pancreatic tissue expressed little to no uPAR or uPA, whereas mice with orthotopic pancreatic tumors (control and pSV-treated) showed strong expression of both uPAR and uPA (expression was stronger at the invasive fronts; Fig. 5A). Mice treated with pU2 showed very low expression levels of both uPAR and uPA (Fig. 5A). In situ TUNEL assay showed that pU2-treated orthotopic tumors showed presence of DNA damage (green fluorescence; Fig. 5A). Determination of percent TUNEL expression indicated that 63% ± 9% cells in pU2-treated tumors had DNA damage whereas no DNA damage was observed in control or pSV-treated mice. We measured tumor volumes by serial sectioning, and we observed that control (550 ± 50 mm³) and pSV-treated (620 ± 100 mm³) mice had the largest tumor volumes while pU2-treated mice had the smallest tumor volume (268 ± 35 mm³); normal mice were used as negative controls (Fig. 5B).

MIA PaCa-2 and PANC-1 cells show colocalization of Lhx-2 and uPA, and simultaneous downregulation of uPAR and uPA in MIA PaCa-2 tumors causes suppression of Lhx-2 in vivo but not in vitro

Fluorescent immunohistochemical analysis for the expression of uPA and Lhx-2 in MIA PaCa-2 and PANC-1 cells revealed that uPA was present in the
cytoplasm, cell membrane, and the nucleus with uPA and Lhx-2 colocalized in the nucleus and cytoplasm (Fig. 6A). Immunohistochemical analysis of Lhx-2 in pancreatic tumor tissues revealed that Lhx-2 is strongly expressed at the invasive front of tumors surrounding the acinar tissues (Fig. 6B). To determine the role of uPA in the nucleus, we performed a transcription factor binding assay using a protein array of known transcription factors. The results show that uPA is bound to the transcription factor Lhx-2 (Fig. 6C). We performed Western blot analysis of nuclear extracts of MIA PaCa-2 and PANC-1 cells transfected with pSV, puPAR, puPA, or pU2 for uPA and Lhx-2 expression. In MIA PaCa-2 cells, transfection with puPAR, puPA, or pU2 caused a decrease in nuclear localization of uPA with a corresponding decrease in the expression level of Lhx-2. In PANC-1 cells, transfection with puPAR, puPA, or pU2 caused a decrease in nuclear uPA similar to MIA PaCa-2 cells but did not result in a decrease in the levels of nuclear Lhx-2 (Fig. 6D). We present a rudimentary theoretical model for the possible role of uPA and Lhx-2 in maintenance of an invasive phenotype and the possible role of Lhx-2 in initiating differentiation (Fig. 6D).

Discussion

In the present study, we have attempted to retard the invasive ability and angiogenic potential of pancreatic cancer cells both in vitro and in vivo. From the human tissue array analysis, we observed that both uPAR and uPA were highly expressed in human pancreatic cancer tissues, and the
expression of uPAR was always correlated with the expression of its ligand uPA. Researchers have shown that activation of the uPAR–uPA system is an early event in the development of pancreatic ductal adenocarcinoma and that uPAR gene amplifications identify a subgroup of particularly aggressive tumors, thereby making the uPAR–uPA system a critical and highly promising target for therapeutic interventions (10).

To target the uPAR–uPA system, we used plasmids expressing shRNA targeting uPAR and uPA (19, 26–30). Using plasmid-mediated RNAi induction did not cause nonspecific inhibition, which indicates that no off-target effects were present. From the Matrigel invasion assay, we observed that the simultaneous downregulation of uPAR and uPA caused a significant decrease in invasive potential as compared to cells downregulated for either uPAR or uPA. Recent research using papillary thyroid carcinoma (PTC) cells provide new functional evidence of the uPAR–uPA system’s role in PTC invasion/metastasis (31). Researchers have also shown that inhibiting the activity of the uPAR and associated molecules like integrins (32) causes a significant decrease of migration and intracellular signaling in cancer cells. The uPAR–uPA system is known to be localized at the invasive front of tumor cells, activating plasmin to plasminogen and further enabling the activation of various proteases (33). Interestingly, the downregulation of the uPAR–uPA system also caused the activation of proapoptotic molecules like caspase 8 and CAD. From our FACS analysis, we did observe the accumulation of cells in the sub-G0/G1 phase in cells downregulated for uPAR and uPA either alone or in combination. Western blot analysis for CAD activation showed that cells simultaneously downregulated for uPAR and uPA had strong CAD activation, indicating that both uPAR and uPA are necessary for maintenance of a viable invasive phenotype.

The classical role of the uPAR–uPA system is the activation of plasmin, and plasmin-overproducing cancer cells are also known to overexpress tPA, uPA, and uPAR. Plasmin is also known to promote angiogenesis and metastasis (34). The mechanisms by which plasmin promotes angiogenesis and metastasis remain poorly understood. We hypothesize that suppressing the plasmin activating system could essentially inhibit angiogenic activation. Our results show that suppression of both uPAR and uPA retarded angiogenic activation under in vitro and in vivo conditions. Our results also show that simultaneous downregulation of uPAR and uPA suppressed angiogenin, a proangiogenic molecule. Recently, researchers have shown that the recombinant kringle domain (UK1) of uPA had antiangiogenic activity by causing the suppression of various proangiogenic molecules, including angiogenin, in glioma cells (35). Our results confirm the previous findings that interference with the uPAR–uPA system retards angiogenesis.

We also observed that the suppression of uPAR–uPA system caused the overexpression of RANTES in both MIA PaCa-2 and PANC-1 cells. Studies have shown that the cleaved form of suPAR binds and activates the fMLP-Rs and regulates the activity of MCP-1, RANTES, and SDF1 receptors (36). Hence, overexpression of uPAR may suppress RANTES expression. RANTES [also known as Chemokine (C-C motif) ligand 5 (CCL5)] is a protein that is encoded by the CCL5 gene in humans. Studies have shown that RANTES plays a role in chemo attraction and activation of immune cells and its potential clinical application as an adjuvant for boosting antitumor immunity has been suggested (37). As our in vivo studies were performed in an immunocompromised setting, further experimentation in a normal setting would shed light on the relevance of RANTES. The regulation of RANTES is not clearly understood and the role of the uPAR–uPA system in modulating RANTES expression has not been reported. It is known that uPAR functionally interacts with integrins, fMLP-receptors (fMLP-Rs), and growth factor receptors, thereby regulating...
cell adhesion, migration, and proliferation. Similarly, interaction of RANTES with uPAR (37) may localize RANTES at the cell surface and not shed into the ECM. Removal of uPAR from the cell surface retards this binding force and allows the shedding of RANTES into the ECM, facilitating the recruitment of immune cells. In vivo experimentation using normal mice (vs immunocompromised mice) would shed light on whether suppression of uPAR would actually recruit immune reaction via RANTES.

In the present study, MCP-1 was also downregulated but only in PANC-1 cells; expression of MCP-1 was not observed in MIA PaCa-1 cells. Similar to RANTES, MCP-1 [monocyte chemotactic protein-1, also known as Chemokine (C-C motif) ligand 2 (CCL2)] is a small cytokine belonging to the CC chemokine family and is known to recruit monocytes (38). The upregulation of RANTES and the downregulation of MCP-1 would indicate the absence of a global cellular immune reaction.

In MIA PaCa-2 cells, ENA-78 expression levels were reduced after uPAR/uPA downregulation but not in PANC-1 cells. ENA-78 (also known as CXCL-5) is produced following stimulation of cells with inflammatory cytokines, such as interleukin-1 or TNF-α, and is known proangiogenic molecule (39–41). Hence, the suppression of ENA-78 would retard angiogenic induction. The link between the uPAR–uPA system and ENA-78 is rudimentary and has not been investigated thoroughly so we only have preliminary evidence indicating the influence of uPAR–uPA system on ENA-78. Similarly, the mechanism by which TIMP-2 levels decrease as a result of suppression of the uPAR–uPA system is not clear. Our Western blot analysis suggests this decrease may be due to the overall shut down of cellular processes and an indicator of apoptosis.

Generally, such responses are accompanied by the expression of OAS1, which was not observed in the present study (supplementary data).

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Our in vivo studies show that the simultaneous down-regulation of uPAR and uPA caused the activation of DNA damage as shown by TUNEL assay. The tumor tissues had decreased tumor cell density and fragmented nuclei, which are both indicative of apoptosis.

Next, to determine the in vitro presence of uPA, we performed immunocytochemistry on both MIA PaCa-2 and PANC-1 cells and looked for the presence of uPA in the nucleus. On further investigation using Western blot analysis, we confirmed its presence in the nucleus of MIA PaCa-2 and PANC-1 cells. To determine whether uPA has any regulatory role, we performed a protein–protein binding array and observed that uPA bound strongly to Lhx-2, a transcription factor. To our knowledge, this is the first report to show the presence of uPA in the nucleus as well as the interaction of uPA with Lhx-2. Modeling and protein–protein docking studies did not indicate any possible interactions, whereas immunoprecipitation studies indicate that Lhx-2 was immunoprecipitated with uPA (see supplementary data).

Recently, researchers have identified Lhx2 as a transcription factor functionally positioned downstream of ¿63 and NF-¿B, but upstream of signals like Wnt/¿-catenin, Bmp, and Shh that are required to drive activated stem cells toward terminal differentiation (42). This would indicate that Lhx-2 may behave like a gatekeeper molecule mediating the activation of cancer stem cells. Immunohistochemical analysis of tumor sections revealed that Lhx-2 was overexpressed at the invasive front of tumors while completely absent in tumors downregulated for both uPAR and uPA. As such, uPA may have certain hitherto unknown regulatory functions, and further study to determine if uPA has any regulatory function is warranted. Taken together, our results show that the targeting of the uPAR–uPA system has significant therapeutic potential for the treatment of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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