Growth Factors, Cytokines, Cell Cycle Molecules

Tissue Plasminogen Activator Induces Pancreatic Cancer Cell Proliferation by a Non-Catalytic Mechanism That Requires Extracellular Signal-Regulated Kinase 1/2 Activation through Epidermal Growth Factor Receptor and Annexin A2

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Tissue plasminogen activator (tPA) is overexpressed in pancreatic ductal carcinoma and is involved in tumor progression. This effect is probably mediated through the activation of angiogenesis, cell invasion, and cell proliferation. Previous studies support the notion that the effects of tPA on cell invasion require its proteolytic activity. Here, we report the molecular mechanism responsible for the proliferative effects of tPA on pancreatic tumor cells. tPA activates the extracellular signal-regulated kinase 1/2 signaling pathway in a manner that is independent of its catalytic activity. We also show that at least two membrane receptors, epidermal growth factor receptor and annexin A2, which are overexpressed in pancreatic cancer, are involved in the transduction of tPA signaling in pancreatic tumors. This observation suggests the establishment of an amplification loop in tumor cell proliferation. Double immunofluorescence experiments showed co-localization of tPA/epidermal growth factor receptor and tPA/annexin A2 in pancreas cancer cells. These results add novel insights into the non-catalytic functions of tPA in cancer and the molecular mechanisms behind the effects of this protease on cell proliferation, including a role for epidermal growth factor receptor. (*Am J Pathol* 2007, 170:1573–1584; DOI: 10.2353/ajpath.2007.060850)

The plasminogen activator system comprises a family of proteins that includes two plasminogen activators, urokinase-type (uPA) and tissue-type (tPA), the zymogen plasminogen, the active form plasmin, and several inhibitors of these proteins. For many years, most studies on the plasminogen system focused on plasmin formation through plasminogen catalytic processing by uPA or tPA. Indeed, many of the effects described for uPA and tPA were accounted for by the broad proteolytic spectrum of plasmin. In particular, for tPA, its main established function is fibrinolysis via plasmin generation. However, recent results have shown that, apart from its proteolytic role, tPA also directly activates intracellular signaling pathways in a non-catalytic manner.^{1–3} These observations have led to the classification of this protease as a cytokine.³ This is not surprising given that the other plasminogen activator, uPA, is involved in the activation of intracellular signals.^{4–8}

tPA and uPA are secreted proteases, and their involvement in the activation of intracellular signal transduction pathways should require their interaction with plasma membrane receptors. The binding of uPA and tPA to membrane receptors has been shown to participate in catalytic and non-catalytic functions of both proteases. In the case of uPA, several lines of evidence show the

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existence of a main specific cellular receptor (uPAR).^{9,10} In contrast, for tPA, several receptors have been described. Annexin A2 (AnxA2) has been identified as a major cell receptor for tPA and plasminogen in endothelial cells,^{11–13} and binding results in a significant increase in the rate of plasminogen activation.^{11,14,15} In neurons, the *N*-methyl-D-aspartate receptor interacts with tPA^{16–18} and participates in tPA signaling.² Recently, Hu et al³ have described that tPA triggers intracellular signal transduction through interaction with the cell membrane receptor LRP-1.

In cancer, the role of plasminogen activators has been extensively studied. Overexpression of uPA and uPAR has been correlated with tumor progression and poor prognosis in several tumor types including breast, lung, colon, and glioma.^{19–23} In contrast, tPA overexpression in tumors is more restricted and has been reported in melanoma, neuroblastoma, and acute leukemia.^{24–29} It has been proposed that the contribution of plasminogen activators to tumor development occurs mainly through the catalytic formation of plasmin, which, in turn, leads to extracellular matrix degradation and cell invasion and migration. Moreover, the induction of cell proliferation may also result from the activation of latent growth factors by plasmin. However, in light of new data indicating non-catalytic functions of tPA and uPA, these mechanisms should be re-evaluated. Indeed, uPA has catalytic-independent mitogenic effects by interaction with uPAR and engagement with several transmembrane receptors including integrins and the epidermal growth factor receptor (EGFR).^{30–32}

Pancreatic ductal adenocarcinoma (PDA) is one of the most devastating tumors and shows low responsiveness to therapy.^{33–35} In our previous work, we have demonstrated that tPA is overexpressed in PDA in comparison to healthy pancreas.³⁶ This finding has been confirmed in unbiased microarray experiments.³⁷ Moreover, tPA is required for *in vitro* invasiveness³⁸ and *in vivo* tumor growth and angiogenesis.^{39,40} We have also found, using a murine model of pancreatic cancer, that the inactivation of tPA is associated with an increase in survival,³⁹ suggesting a relevant role of this protease in tumor progression. This effect is associated with a marked reduction in cell proliferation in ductal tumors as well as with reduced angiogenesis. It has recently been reported that tPA mediates the invasion of pancreatic cancer cells *in vitro* by interaction with AnxA2 and local plasmin formation.⁴¹ However, the molecular mechanisms governing the proliferative response to tPA in this tumor remains to be elucidated. In addition, the role of the catalytic and non-catalytic functions of this protease in cancer has not been analyzed. Here, we demonstrate that the mitogenic effects of tPA on pancreas cancer cells are mediated by extracellular signal-regulated kinase 1/2 (ERK1/2) signal pathway activation independently from the catalytic activity of this protease. We also analyzed the membrane receptors involved in this event. Using specific small-interference RNA (siRNA) and pharmacological inhibitors, we show that both EGFR and AnxA2 are required for tPA signaling. These findings support the

notion that, similarly to uPA, multiprotein membrane complexes involving the EGFR may participate in tPA-induced intracellular signaling in pancreatic cancer cells.

Materials and Methods

Materials

All materials were from Sigma Chemical Corp. (St. Louis, MO) unless otherwise stated. Two recombinant tPA preparations were used: human tPA (Actilyse; Boehringer Ingelheim, Barcelona, Spain) and murine tPA (Loxo Laboratories, Dossenheim, Germany). For catalytically inactive tPA we used human tPA S478A (a kind gift from Genentech, San Francisco, CA) or mouse tPA S478A (Loxo Laboratories).

Antibodies

The following primary antibodies were used: AnxA2 mouse monoclonal antibody (Transduction Laboratories, Kensington, KY), AnxA2 polyclonal antiserum raised in our laboratory using recombinant human AnxA2,³⁹ goat anti-tPA polyclonal antibody (387) (American Diagnostica, Greenwich, CT), rabbit polyclonal antibody against activated ERK1/2 (phospho-p44/42 MAP kinase; Cell Signaling Technology, Danvers, MA), rabbit polyclonal antibody against total ERK1/2 (Upstate Biotechnology International, Lake Placid, NY), rabbit polyclonal antibodies against phosphorylated and total JNK, AKT, and EGFR (Cell Signaling Technology), and anti- α -tubulin mouse monoclonal antibody DM 1A (Sigma Chemical). As secondary antibodies, fluorescein isothiocyanate-, alkaline phosphatase-, and peroxidase-conjugated antibodies recognizing rabbit, mouse, or goat Ig were purchased from Dako (Glostrup, Denmark). For immunofluorescence experiments, we used goat anti-rabbit Ig coupled to Alexa 488 to detect AnxA2 and EGFR. Biotinylated donkey anti-goat Ig (Jackson Laboratories, Bar Harbor, ME) followed by streptavidin linked to rhodamine was used for the detection of tPA.

Cell Culture

PANC-1, Hs766T, SK-PC-1, and BxPC3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL), nonessential amino acids, penicillin, and streptomycin. Immortalized human pancreatic ductal epithelial (HPDE) cells were obtained from Dr. M. Tsao (Ontario Cancer Institute, Toronto, ON, Canada) and cultured in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract (Life Technologies, Grand Island, NY), as described elsewhere.⁴² Chinese hamster ovary (CHO) cells transfected with control plasmid (CHO-K1) or with a plasmid coding for the human EGFR cDNA (WT-EGFR-CHO) were a gift from Dr. S. Gonias (University of California, San Diego, CA).³¹ WT-EGFR-CHO cells were cultured in DMEM (high glucose)

with 10% FBS (GIBCO-BRL), penicillin, streptomycin, and nonessential amino acids. CHO-K1 cells were cultured in DMEM/F-12 (1:1) with 10% FBS (supplemented with penicillin, streptomycin, and nonessential amino acids). Cells were maintained at 37°C in a 5% CO₂ atmosphere.

Cell Treatments

To analyze signaling pathway activation and proliferation, cells were grown to confluence in complete medium and starved for 2 to 3 days using culture medium without FBS or growth supplements. tPA (0.5, 2, and 5 µg/ml) was then added for variable periods of time as indicated in the text. U0126 (10 µmol/L) and PD98059 (30 µmol/L), inhibitors of mitogen-activated protein kinase kinase (MEK)1/2 and MEK1, respectively, were added 15 minutes before tPA treatment. Tyrphostin AG1478 (10 µmol/L), an inhibitor of EGFR, was added 30 minutes before the addition of tPA.

Cell Proliferation Assays

Cells were seeded in 24-well plates in complete medium and cultured until confluent. After transfer to DMEM without FBS for 48 hours (Hs766T) or keratinocyte serum-free medium without any supplements (HPDE), wild-type or mutant tPA S478A and [³H]thymidine (1 µCi/ml/well) (GE Healthcare, Buckinghamshire, UK) were added for 24 hours. Labeled DNA was precipitated with 10% trichloroacetic acid, cells were lysed in 0.5 mol/L NaOH, and 1 mol/L HCl was added. The radioactivity in the precipitate was measured by liquid scintillation counting. All experimental conditions were tested in triplicate, and at least three independent experiments were performed.

Immunohistochemistry

Samples of pancreatic cancer tissues were retrieved from the files of the Department of Pathology, Hospital del Mar, Barcelona, Spain. All records, corresponding to patients from whom pancreatic tissue was obtained between 1996 and 2000 during a surgical intervention, were retrieved and examined. These procedures were approved by the Ethical Committee for Clinical Research of our Institution. Immunohistochemical analyses were performed using 5-µm sections of formalin-fixed, paraffin-embedded tissue blocks. Antigens were retrieved by immersing slides in 10 mmol/L citrate, pH 7.3, at 120°C for 1 minute in an autoclave. For EGFR detection using immunoperoxidase, a kit from Dako, optimized for formalin-fixed, paraffin-embedded tissues, was used in a TechMate 500 automated immunostainer (Ventana Medical System, Tucson, AZ). Primary antibodies were added for 30 minutes. As secondary antibody, the Envision⁺ anti-rabbit reagent was applied (Dako). Reactions were developed using diaminobenzidine as chromogenic substrate. Sections were counterstained with hematoxylin, dehydrated, and mounted. Sections were visualized in an Olympus AX70 microscope (Tokyo, Japan), and images

were acquired using Studio Lite 1.0 software (Olympus). Images were obtained with the 20× objective. For the immunofluorescence experiments, tissues were snap-frozen in OCT and stored at -80°C until used. Sections (5 µm) were fixed in ice-cold acetone for 10 minutes. Primary antibodies were added overnight at 4°C. Biotinylated secondary antibodies were added for 1 hour at room temperature. Streptavidin-rhodamine was added for 15 minutes. Immunofluorescent images were acquired with a Leica DMRB microscope adapted to a DC300F camera (Leica Lasertechnik GmbH, Mannheim, Germany) fitted with appropriate filters. Images were obtained with a 40× objective. As negative controls, tissues were incubated with nonimmune (Dako) or preimmune rabbit serum. All sections were checked by a pathologist (J.M.C.) with extensive experience in pancreatic diseases.

Immunoprecipitation, Pull-Down Assays, and Western Blotting

Cells were rinsed in ice-cold phosphate-buffered saline (PBS), pH 7.2, and lysed at 4°C in 67 mmol/L Tris, pH 6.8, and 2% sodium dodecyl sulfate for total extracts or in buffer A [50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, 0.1% Triton X-100 plus protease and phosphatase inhibitors (200 mmol/L Pefabloc, 1 mmol/L aprotinin, 20 µmol/L leupeptin, 0.1 mmol/L sodium vanadate, 1 mmol/L sodium fluoride, and 10 mmol/L sodium pyrophosphate)] for immunoprecipitation analysis. Protein concentration was measured using Bradford or Lowry assay reagents (Bio-Rad Laboratories, Hercules, CA). Immunoprecipitation and pull-down assays were performed using 0.4 to 1 mg of cellular proteins. Lysates were precleared using 20 µl of protein G-Sepharose beads. The supernatant was incubated with anti-AnxA2 monoclonal antibody (0.5 µg/ml) for 3 hours at 4°C. After centrifugation, protein G-Sepharose blocked with bovine serum albumin (BSA) was added, and beads were washed three times in buffer A. For pull-down studies, tPA was coupled to cyanogen bromide-activated Sepharose 4B, following the manufacturer's instructions. Cell lysates were incubated with 20 µl of tPA-Sepharose (1:1, v/v) or with Sepharose alone, in buffer A for 1 hour at 4°C. After centrifugation, unbound proteins were collected and bound proteins were washed three times with buffer A. Laemmli sample buffer was added to washed Sepharose beads, and samples were heated to 95°C for 5 minutes. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters for Western blot analysis. After incubation with the appropriate primary antibody, species-specific secondary antibodies conjugated to horseradish peroxidase and the enhanced chemiluminescence method (Amersham) were used.

Flow Cytometry Analysis

To determine the presence of AnxA2 in the outer side of the plasma membrane, flow cytometry was used. Pancre-

atic cancer cells (PANC-1 and Hs766T) were resuspended at 10^6 cells/ml in PBS with 2% BSA for 20 minutes at room temperature. Cells were then incubated with rabbit anti-AnxA2 antibody or with an irrelevant rabbit IgG for 1 hour at 4°C, washed four times with PBS with 2% BSA, and incubated with fluorescein isothiocyanate-conjugated anti-rabbit Ig for 30 minutes at 4°C. Cells were analyzed in a FACScan (Becton, Dickinson and Company, San Jose, CA).

Enzyme-Linked Immunosorbent Assay

Hs766T cells were cultured in 96-well Nunclon plates (Nunc, Naperville, IL) in complete medium. When cells reached confluence, they were starved for 24 hours in DMEM without FBS. Cells were equilibrated with incubation buffer [11 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 137 mmol/L NaCl, 4 mmol/L KCl, 3 mmol/L $MgCl_2$, 1 mmol/L glucose, and 5% BSA] for 30 minutes at 37°C and incubated for 15 minutes at 37°C with purified rabbit anti-AnxA2 Ig or with normal rabbit Ig, both obtained by ammonium sulfate precipitation. After three washes with incubation buffer, tPA (50 ng/ml) was added for 30 minutes at 37°C; cells were fixed with methanol, washed three times with PBS-0.02% Tween 20 (PBST), and incubated with goat anti-tPA for 1 hour at 37°C. After washing with PBST, alkaline phosphatase-coupled anti-goat Ig was added for 1 hour at 37°C. Cells were incubated with 4-methylumbelliferyl phosphate (1 mg/ml in triethanolamine buffer, pH 9.5) for 20 minutes at room temperature, and enzymatic activity was determined using a Cytofluor 235 instrument (Millipore, Bedford, MA).

siRNA Assays

siRNA knockdown was performed using synthetic double-stranded RNA purchased from Xeragon (Huntsville, AL). The sequence used to target AnxA2 was 5'-AUGU-CUACUGUUCACGAAUCCGC-3' (siAnxA2), and an unrelated oligonucleotide (siControl) recognizing an irrelevant transcript was used as a negative control. Hs766T cells were cultured in 24-well plates up to 30% confluence and transfected using TransIT-TKO transfection reagent (Mirus, Madison, WI), following the manufacturer's instructions. In brief, TransIT-TKO transfection reagent (2.5 μ l) was added dropwise into 50 μ l of Opti-MEM (GIBCO-BRL) and incubated at room temperature for 20 minutes. The siRNA oligonucleotides were then added at a final concentration of 25 nmol/L and incubated for 20 minutes at room temperature. The reagent/siRNA mixture (50 μ l) was added to cells in 250 μ l of complete medium. After 24 hours, the medium was replaced with fresh complete medium. The effects of treatment with siRNA were followed for 1 to 8 days and analyzed by Western blotting.

To analyze the effects of siRNA on ERK1/2 activation and cell proliferation, HPDE cells were transfected with siRNA (AnxA2 or Control) and 48 hours later, medium was replaced by keratinocyte serum-free medium without

any supplements for 24 hours. tPA (5 μ g/ml) or growth factors (EGF and bovine pituitary extract) were then added for 5 minutes (ERK1/2 activation) or 24 hours (cell proliferation).

Statistical Analysis

The effect of the treatments was compared with the control experimental conditions using Student's *t*-test. Values were considered statistically significant when $P < 0.05$.

Results

Pancreatic Cancer Cell Proliferation Induced by tPA Is Mediated by Activation of ERK^{MAPK} and Is Independent of tPA Proteolytic Activity

tPA has a mitogenic effect on a variety of cell types, including pancreatic cells.^{40,43,44} However, the molecular mechanisms involved in this effect are unknown. First, we analyzed the effects of tPA on the growth of two pancreatic cell lines: Hs766T, a cell line derived from a human PDA,⁴⁵ and HPDE, a nontumorigenic immortalized pancreatic ductal cell line.⁴² tPA induced a proliferative response, detected as an increase in [³H]thymidine uptake, in both cell lines (Figure 1A). Because the ERK1/2 pathway plays a major role in the control of cell proliferation, we examined the capacity of tPA to activate this pathway by means of Western blotting with antibodies specific for phosphorylated or total ERK1/2. Treatment with FBS (Hs766T) or growth supplements (HPDE) was used as a positive control. tPA induced an early increase in phospho-ERK1/2 levels (2 to 5 minutes after its addition to cells) and this activation was maintained for 15 minutes. Erk1/2 activation was reduced to control levels when tPA treatment was performed in the presence of MEK inhibitors U0126 or PD98059 (data not shown), indicating the specificity of the pathway. Treatment with U0126 or PD98059 alone did not change basal levels of phospho-ERK1/2. Total levels of ERK1/2 protein were unaffected (Figure 1B, bottom panels). We also analyzed the involvement of other signal transduction pathways in response to tPA treatment. As shown in Figure 1C, in HPDE cells, a very slight and transient increase in AKT phosphorylation was observed after a 2-minute treatment with tPA, whereas activation of the JNK and p38 pathways was not detected. These data indicate that tPA exerts a predominant effect on the phosphorylation of the ERK1/2 pathway. To determine whether the proliferative effects of tPA on pancreatic cancer cells are mediated by the observed activation of the ERK1/2 pathway, we analyzed [³H]thymidine uptake in Hs766T cells after tPA treatment in the presence of U0126 and PD98059, two well-characterized inhibitors of this pathway. Figure 1D shows that both inhibitors decreased tPA mitogenic response to a level similar to that of untreated cells, indicating that the proliferative effects of tPA are mediated by the activation of the ERK1/2 pathway. To study whether the phosphorylation of ERK1/2 and cell proliferation was

Figure 1. tPA induces the activation of the ERK1/2 pathway mediating pancreatic cell proliferation in a non-catalytic manner. **A:** Hs766T and HPDE cells were deprived of FBS (Hs766T) or growth supplements (HPDE) for 48 hours. tPA was added at the concentrations indicated, and [³H]thymidine uptake was determined after 24 hours. tPA induced an increase in cell proliferation in both cell lines (**P* < 0.05). Untreated cells (–) or cells treated with 10% FBS (Hs766T) or growth supplements (HPDE) (+) were used as negative and positive controls, respectively. **B:** Hs766T and HPDE cells were serum- or growth supplement-starved for 48 hours and then treated with 5 μg/ml tPA for variable periods of time. Cells were collected and lysates were analyzed by Western blotting using antibodies detecting phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK1/2). Five minutes of treatment with 10% FBS (Hs766T) or growth supplements (HPDE) was used as positive control for ERK1/2 activation (+). **C:** HPDE cells were grown to confluence, deprived of growth supplements for 48 hours, and then treated with 5 μg/ml tPA for times from 2 to 15 minutes. Cells were collected and lysates were analyzed by Western blotting using antibodies against phosphorylated and total AKT, JNK, and p38. No significant effects were observed on JNK and p38 signaling pathways, whereas a slight activation of AKT was detected after 2 minutes of treatment with tPA. Treatment (5 minutes) with growth supplements was used as positive control (+). **D:** Hs766T cells, cultured as indicated before, were preincubated with U0126 (10 μmol/L) or PD98059 (30 μmol/L) for 150 minutes before treatment with tPA (2 μg/ml). [³H]thymidine uptake was measured 24 hours later as indicated in Materials and Methods. These two inhibitors blocked the mitogenic effect of tPA, although only the effect of PD98059 was statistically significant (**P* < 0.05). **E:** HPDE cells were cultured as indicated above and left untreated (–) or treated with growth supplements (+) or with wild-type (tPA) or catalytically inactive tPA (tPA S478A) (5 μg/ml) as described in Materials and Methods. ERK1/2 phosphorylation was determined using Western blotting. ERK1/2 activation on treatment with mutant tPA indicates that the effects are independent from the proteolytic activity of tPA. For cell proliferation studies, [³H]thymidine uptake was measured after adding wild-type or catalytically inactive tPA at 2 and 5 μg/ml. Untreated cells (–) or cells treated with growth supplements (+) were used as negative and positive controls, respectively. Both wild-type and mutant protein induced an increase in cell proliferation. This effect was statistically significant (**P* < 0.01). These data indicate that tPA effects in cell proliferation are independent of its catalytic activity. Three independent experiments were performed for each point.

dependent on the enzymatic activity of tPA, we used a mutant protein (tPA S478A) lacking proteolytic activity as a result of the substitution of a critical Ser residue at the catalytic site. tPA S478A reproducibly increased the levels of phospho-ERK1/2 to an extent similar to that of catalytically active tPA (Figure 1E, top panel). In addition, treatment of cells with plasmin alone did not activate the ERK1/2 pathway (data not shown). [³H]Thymidine uptake in HPDE cells showed that both wild-type and mutant tPA S478A induce a similar increase in cell proliferation (Figure 1E, bottom panel). Taken together, these results indicate that tPA signaling through the ERK1/2 pathway, and the subsequent proliferative response, do not require proteolysis or plasmin formation.

EGF Receptor and AnxA2 Are Overexpressed and Co-Localize in Human Pancreatic Tumors and Cell Lines

tPA is a secreted protein, and it is conceivable that the signaling effects of this protease require interaction with a plasma membrane receptor. Therefore, we explored the role of two putative membrane receptors in the ERK1/2 activation induced by tPA, EGFR, and AnxA2. EGFR integrates signals from various types of cytokines/growth factors, is classically related to ERK1/2 signal pathway activation,⁴⁶ and is overexpressed in PDA.⁴⁷ AnxA2 has been described to interact with tPA in pancreatic cancer cells and to mediate *in vitro* invasion.⁴¹

First, we examined EGFR and AnxA2 expression in several pancreatic cell lines by Western blotting. EGFR levels ranged from high (BxPC3, HPDE) to moderate (SK-PC-1, PANC-1) and low (Hs766T) in the cell lines analyzed. In contrast, AnxA2 was abundant in all cell lines (Figure 2A). The expression of EGFR^{47–52} and AnxA2^{41,53–55} have been analyzed separately in a few reports. We then examined EGFR and AnxA2 expression in a panel of human PDA by immunohistochemistry, observing overexpression of both proteins in tumors in comparison to normal pancreas. Using paraffin-embedded tissue, EGFR was not detected in normal pancreas (Figure 2B, a) but was overexpressed in pancreatic intraepithelial neoplasia (PanIN) (Figure 2B, b) and in invasive pancreatic tumors (Figure 2B, c). In contrast, AnxA2 was undetectable in acinar cells but was expressed by normal ductal cells, mesenchymal cells, and vascular endothelium in normal pancreatic tissue (Figure 2B, d). In ductal cells, AnxA2 was detected in the apical membrane (Figure 2B, d, arrowheads). In PanIN and pancreatic adenocarcinomas, AnxA2 was almost universally overexpressed (11/14 PanIN-2 and 8/9 PanIN-3, 14/16 PDA), and was distributed throughout the entire plasma membrane (Figure 2B, b and c, arrowheads), in agreement with the loss of polarity associated with epithelial cell transformation. Therefore, AnxA2 overexpression is a common and early event in pancreatic carcinogenesis. We analyzed the co-localization of tPA and EGFR or AnxA2 by double immunofluorescence on frozen tissue sections. Figure 2C shows that EGFR and tPA (panels a–c) or AnxA2 and tPA (panels d–f) co-localized in tumor

Figure 2. EGFR and AnxA2 are overexpressed in human pancreatic cell lines and tumors. **A:** EGFR and AnxA2 expression was analyzed in total lysates of cultured pancreas cancer cells (BxPC-3, HPDE, Hs766T, PANC-1, SK-PC-1) using Western blotting. Tubulin was used as a loading control. **B:** Sections of formalin-fixed paraffin-embedded pancreatic tissues corresponding to normal pancreas (**a** and **d**), PanIN (**b** and **e**), and invasive tumor (**c** and **f**) were analyzed for EGFR and AnxA2 expression as described in Materials and Methods. Overexpression of both proteins (**arrow-heads**) was detected in PanIN lesions (**b** and **e**) and in tumor samples (**c** and **f**). **C:** Frozen sections of tumoral pancreatic tissues were analyzed by double immunofluorescence for tPA and EGFR expression (**a** and **b**) and for tPA and AnxA2 expression (**d** and **e**) as described in Materials and Methods. The overlay of both signals is shown in the merge panels (**c** and **f**). Scale bars = 200 μ m.

Figure 3. EGFR is required for the induction of ERK1/2 activation by tPA. **A:** EGFR was associated to tPA by pull-down analysis. HPDE cell lysates were incubated with tPA-Sepharose or BSA-Sepharose and EGFR in the unbound (U) or bound (B) fractions was detected by Western blot analysis. Pyruvate kinase (PK) was used as negative control. **B:** HPDE cells were grown to 70% confluence, deprived of growth supplements for 48 hours, and then treated with 5 μ g/ml tPA for 2 to 15 minutes in the absence or in the presence of the EGFR inhibitor tyrphostin AG1478 (10 μ mol/L). Cells were collected and lysates were analyzed by Western blotting using antibodies against phosphorylated (P-ERK1/2) and total ERK1/2 (ERK1/2). Treatment with growth supplements (+) was used as positive control for ERK1/2 activation. Inhibition of EGFR results in abolishment of tPA signaling. **C:** Expression of AnxA2 and EGFR in control CHO cells (K1-CHO) and in cells transfected with a plasmid coding for EGFR (WT-EGFR-CHO) (left panel). Cells were grown to confluence, serum-starved for 48 hours, and then treated with 5 μ g/ml tPA for 2 to 15 minutes. Lysates were analyzed for ERK1/2 activation by Western blotting. Treatment with 10% FBS was used as positive control (+). tPA activated the ERK1/2 pathway only in WT-EGFR-CHO cells, proving the relevance of this receptor in mediating the effects of tPA.

cells of PDA. These findings support the notion that these proteins are coexpressed in pancreatic cancer cells and thus play crucial roles in tPA functions *in vivo*.

EGFR Binds to tPA and Is Required for tPA-Induced ERK1/2 Signaling

To determine whether tPA and EGFR are associated in cells, extracts from HPDE cells expressing high levels of EGFR (Figure 2A) were incubated with tPA-coupled Sepharose, and bound proteins were detected by Western blotting. Figure 3A shows that EGFR specifically bound tPA-Sepharose but not BSA-Sepharose. Pyruvate kinase, a cytosolic protein, did not bind to tPA, supporting the specificity of the interaction. We then analyzed the involvement of EGFR in tPA signaling in pancreatic cells. For this purpose, HPDE cells were treated with tPA in the presence or absence of the selective EGFR chemical inhibitor AG1478 and the effects on ERK1/2 phosphorylation were determined. ERK1/2 activation was completely abolished in the presence of AG1478 and this effect was specific for tPA, since no effect was observed on growth supplement-induced ERK1/2 activation (Figure 3B). To assess further the role of EGFR in the tPA-induced ERK1/2 activation, we used CHO cells transfected with the human EGFR cDNA (wt-EGFR-CHO) or with a control plasmid (CHO-K1).³¹ As shown in Figure 3C, left panel, both cell lines expressed AnxA2. tPA induced activation of ERK1/2 only in CHO cells expressing the human EGFR (Figure 3C, right panel). This effect was

Figure 4. tPA binds to AnxA2 in cultured pancreatic cells. **A:** AnxA2 pull-down assays using tPA-coupled Sepharose. Pancreatic cancer cell lysates were incubated with tPA-Sepharose (tPA) or BSA-Sepharose (BSA), and the presence of AnxA2 in the bound (B) and unbound (U) fraction was determined using Western blotting. E-cadherin detection was used as a control of the interaction specificity. AnxA2 was specifically detected in the bound fraction of tPA-Sepharose. **B:** Coimmunoprecipitation of endogenous tPA and AnxA2 in pancreas cancer cells. Lysates of pancreatic cancer cells expressing high levels of tPA (SK-PC-1) or lacking tPA expression (PANC-1) were used to immunoprecipitate AnxA2. The presence of AnxA2 and tPA in cell lysates (L) and immunoprecipitates (IP) was analyzed by Western blotting. Coimmunoprecipitation of the two proteins was detected in tPA-expressing SK-PC-1 cells. **C:** Flow cytometry analysis of AnxA2 expression in Hs766T cells. Living cells were incubated with rabbit anti-AnxA2 Ig or an irrelevant IgG and the intensity of membrane staining was measured by fluorescence flow cytometry. **D:** tPA binding to membrane AnxA2 detected by enzyme-linked immunosorbent assay. Confluent Hs766T pancreatic cancer cells were cultured as described in Materials and Methods, incubated in the presence of an antibody against AnxA2 (α -AnxA2) or an irrelevant antibody (Control Ig), and recombinant tPA was added (50 ng/ml). Membrane-bound tPA was detected by enzyme-linked immunosorbent assay. Data shown correspond to the results of one of the three independent experiments performed and values correspond to mean $\pm$ SD of triplicates. Anti-AnxA2 antibodies inhibit the binding of tPA to Hs766T cells by 77% in comparison to an irrelevant rabbit Ig (Control Ig) (* P < 0.05).

also specific for tPA, as FBS induced strong ERK1/2 activation in both cell lines. These results indicate that EGFR modulates the cellular response to tPA.

AnxA2 Binds tPA and Is Required for tPA-Induced ERK1/2 Signaling in Pancreatic Cancer Cells

AnxA2 is a receptor for tPA in endothelial and promyelocytic leukemia cells,^{11,12,56} and an interaction of tPA with AnxA2 to enhance plasmin activation has recently been described in pancreatic cancer cells.⁴¹ To determine whether AnxA2 is involved in the signaling response to tPA, we used several approaches. First, we analyzed the interaction of cellular AnxA2 and tPA by pull-down assays using tPA-Sepharose and lysates from BxPC-3 and PANC-1 cells. AnxA2 was specifically detected in the bound fraction of tPA-Sepharose but not in the BSA-Sepharose fraction (Figure 4A). In contrast, E-cadherin, a membrane protein that is strongly expressed in these cells, was undetectable in the tPA-Sepharose bound fraction, thereby indicating the specificity of the pull-down assay (Figure 4A, bottom panel). Second, the in-

teraction between tPA and AnxA2 in pancreatic cells was confirmed using immunoprecipitation analysis. Cell lysates from SK-PC-1 and PANC-1 pancreatic cancer cells were incubated with anti-AnxA2 antibodies and the presence of tPA levels in the immunoprecipitated fraction was tested by Western blotting (Figure 4B). Furthermore, tPA was co-immunoprecipitated with AnxA2 from lysates of SK-PC-1 cells expressing high levels of endogenous tPA but not from PANC-1 cells lacking tPA expression (Figure 4B). When a control nonimmune IgG was used for the immunoprecipitation, tPA was not detected by Western blotting, thereby supporting the specificity of these findings (data not shown). Because AnxA2 is present in the cytoplasm and in the plasma membrane and distinct functions have been assigned to each fraction, we analyzed the contribution of plasma membrane-associated AnxA2 to tPA binding. We observed that AnxA2 was expressed in the plasma membrane of Hs766T by flow cytometry analysis on living cells (Figure 4C). Furthermore, using an enzyme-linked immunosorbent assay, anti-AnxA2 antibodies blocked tPA binding to Hs766T cells by 77%, whereas a control immunoglobulin had no effect (Figure 4D). Similar results were obtained using PANC-1 cells (data not shown). These results indicate that plasma membrane AnxA2 is involved in the binding of tPA to pancreatic cancer cells.

We then explored the hypothesis that tPA binding to AnxA2 is required for the activation of the ERK1/2 signaling pathway, as observed for EGFR. Double-stranded RNA corresponding to nucleotides 1–22 of AnxA2 (siAnxA2) was transfected into HPDE cells. We observed a 70% reduction in total cellular levels of AnxA2 up to 6 days after transfection (Figure 5A). These effects were specific, as the siAnxA2 did not affect the levels of α -tubulin. Furthermore, neither did the treatment with a double stranded oligonucleotide targeting an irrelevant transcript (siControl) influence AnxA2 levels. Figure 5B shows the activation of ERK1/2 after siRNA transfection in HPDE cells on treatment with tPA. Although the transfection with an irrelevant siRNA (siControl) had no effect on the activation of ERK1/2, transfection with siAnxA2 resulted in a complete abolishment of tPA-induced ERK1/2. In contrast, activation of ERK1/2 by FBS and total levels of ERK1/2 were unaffected by either siRNA treatment (siAnxA2 or siControl), which indicates the specificity of AnxA2 interference in the tPA-induced signaling.

As we have demonstrated earlier (Figure 1C), the mitogenic effects of tPA are mediated by ERK1/2 activation. Therefore, we analyzed whether AnxA2, in addition to participating in tPA-triggered ERK1/2 activation, was also required for tPA-induced growth stimulation. The proliferative response of HPDE cells to tPA, detected as an increase in [3 H]thymidine uptake, was significantly reduced after transfection with siAnxA2, whereas transfection with an irrelevant siRNA (siControl) showed no effect (Figure 5C). Similar results were obtained using 5-bromo-2'-deoxyuridine uptake (data not shown). Taken together, these results indicate that AnxA2 is a functional tPA receptor that participates in the induction of cell proliferation and signaling by tPA in pancreatic cancer cells.

Figure 5. AnxA2 mediates ERK1/2 activation and cell proliferation induced by tPA in HPDE cells. **A:** Inhibition of AnxA2 expression using siRNA. HPDE cells were transiently transfected with siRNA against AnxA2 (siAnxA2) or an irrelevant target (siControl) as indicated in Materials and Methods. A selective depletion of AnxA2 was achieved for up to 6 days, as detected by Western blotting. Tubulin levels in cell lysates were assayed as a control. Quantification of the reduction of AnxA2 levels is shown in the graph. **B:** Depletion of AnxA2 using siRNA blocks tPA-induced ERK1/2 phosphorylation. HPDE cells were transfected with siRNA targeting AnxA2 (siAnxA2) or an irrelevant target (siControl); after 48 hours, medium was replaced with keratinocyte serum-free medium, and 24 hours later, cells were left untreated (–) or treated with 5 μ g/ml tPA for 5 minutes (tPA) or with growth supplements for 5 minutes (+). Phosphorylated-ERK1/2 (P-ERK1/2) and total ERK1/2 (ERK1/2) levels were determined by Western blotting. **C:** Depletion of AnxA2 by siRNA blocked cell proliferation induced by tPA. HPDE cells untreated (–) or transfected with siRNA corresponding to AnxA2 (siAnxA2) or an irrelevant target (siControl) were cultured and treated with tPA (5 μ g/ml) and cell proliferation was assayed by [3 H]thymidine uptake. Cells treated with growth supplements were used as positive control (+). Results shown are representative of three independent experiments. Transfection with anti-AnxA2 siRNA (siAnxA2) blocked the mitogenic effect of tPA, whereas no effect was observed when siRNA against an irrelevant target (siControl) was used (* P < 0.05).

Discussion

A thorough understanding of the molecular mechanisms involved in the growth and invasive properties of PDA is crucial to decrease the mortality caused by this tumor. Because the disease progresses very rapidly,³⁴ studies into the mechanisms involved in the early steps of carcinogenesis and the factors that modulate the response to therapy are urgently required.³⁵ Identification of new targets, which might include proteins involved in tumor

growth and signaling pathways, will then be one of the main focuses of new discoveries in pancreatic cancer biology.^{57–61}

In recent years we have shown that tPA is overexpressed in PDA and is involved in *in vitro* invasion^{36,38} and in tumor progression using genetic mouse models.³⁹ This tPA overexpression is unlike many other tumor types in which the uPA system has been proposed to play a predominant role. Overall, there is evidence indicating that the effects of tPA on pancreatic cancer takes place at three levels: cell proliferation, invasion, and angiogenesis.^{39–41} The pleiotropic effects of this protein indicate that tPA could be a new therapeutic target for pancreatic cancer. Although a few studies have addressed the mechanisms through which tPA may participate in invasion and angiogenesis,^{38,40,41} little is known about the molecular mechanisms involved in tPA-mediated cell proliferation. Here, we provide the first evidence that tPA induces pancreatic cancer cell proliferation through the activation of the ERK1/2 signaling pathway and, most importantly, that this effect is independent of tPA catalytic activity and mediated by AnxA2 and EGFR.

tPA enhances cell proliferation in a variety of cellular systems,^{1,62–66} including pancreatic cancer,^{38–40} although the molecular mechanism underlying this effect has not been well characterized. Maupas-Schwalm et al¹ reported that the mitogenic effects of tPA on smooth muscle cells require the activation of Src and ERK1/2 and are independent from its catalytic activity. Although we observed a slight increase in AKT phosphorylation, the major effect of tPA on PDA cells was an induction of ERK1/2 activation in a non-catalytic-dependent manner, consistent with the effects of tPA on cell proliferation. tPA catalytic-independent activation of the ERK1/2 pathway and subsequent cell proliferation induction is important for understanding the role of this protease in pancreatic cancer progression. Indeed, the effects of tPA seem to be context-dependent; in human fibroblasts and endothelial cells, proteolytic activity is not required for proliferation,^{1,62,63} whereas in vascular smooth muscle cells,⁶⁶ mouse fibroblasts,⁶⁵ and hepatocytes,⁶⁴ proteolytic activity is required for this event. The complexity of signaling by tPA is also underlined by the finding that, in fibroblasts, tPA induces matrix metalloproteinase-9 gene expression in a non-catalytic manner through the activation of the ERK1/2 pathway.³ However, the effects of tPA on pancreatic cancer cell invasion *in vitro* require plasmin generation at the cell surface.⁴¹ Therefore, further research is necessary to dissect fully the proteolysis-dependent and -independent effects of tPA to develop specific therapeutic strategies.

Activation of ERK1/2 by tPA independent of its catalytic activity implies the binding of the secreted protein to a cell membrane receptor. Using pharmacological inhibitors and siRNA, we have shown that two cellular receptors, AnxA2 and EGFR, are involved in tPA-induced signaling. Both proteins are overexpressed in pancreatic cancer^{39,41,47–55} and display co-localization in tumor tissue, suggesting the establishment of an amplification loop that may contribute to the effects of tPA on tumor progression. AnxA2 has previously been described as a

tPA receptor in endothelial cells,¹² where it increases the catalytic activity of tPA. In addition, it has been recently reported that similar effects take place in pancreatic cancer cells by activating local plasmin formation and tumor cell invasion.⁴¹ However, a role for AnxA2 in the non-proteolysis-dependent ERK1/2 activation and proliferation effects of tPA has not been shown previously. Together with prior studies, our findings indicate a pivotal contribution of AnxA2 to the enhancement of proliferation and invasion by tPA, regardless of the requirement for proteolytic activity. Because tPA also increases endothelial cell proliferation,^{63,66} it is conceivable that AnxA2 is also an important mediator of angiogenesis in a paracrine manner.³⁹

The finding that EGFR is required for tPA-mediated signaling is particularly important because this receptor integrates a wide variety of ligands,⁴⁶ is overexpressed in pancreatic cancer, and co-localizes with AnxA2 and tPA. Furthermore, EGFR is a major drug target, the potential of which is currently being explored in pancreatic cancer.^{67–69} The expression of a high number of receptor molecules on the surface of tumor cells is thought to be a general mechanism to increase their sensitivity to low concentrations of growth factors, thereby allowing the autonomous growth of cancer cells. Among the growth factor receptors involved, members of the EGFR family (comprising EGFR/ErbB1, ErbB-2, ErbB-3, and ErbB-4) have a central role in the pathogenesis and progression of a wide variety of tumors,^{70,71} including pancreatic cancer.^{48–52} The EGF ligand/receptor system constitutes an integrated complex in which a signal from an individual receptor is often transmitted to other receptors of the same family, thereby leading to amplification and diversification of the initial signal, a critical phenomenon for cell transformation. Furthermore, it has been reported that the type and duration of EGFR signaling is determined by the identity of the ligand, composition of the receptor complex, and specific structural determinants of the receptors, including the presence of activating mutations.⁶⁶ uPA, the other main plasminogen activator, also induces cell proliferation by activation of ERK1/2 through a multimolecular complex containing EGFR, $\alpha 3\beta 1/\alpha 5\beta 1$ integrins, and focal adhesion kinase.^{30,72} It is attractive to speculate, on the basis of the present data, that the proliferative and signaling effects induced by tPA in pancreatic cancer cells also require a multiprotein complex in which at least AnxA2 and EGFR are involved: AnxA2, which shows strong affinity for tPA^{12,73} but has no transmembrane domain, could be the initial partner for tPA in the membrane, and this interaction could in turn engage EGFR, acting as a canonical signal transducer molecule. Ongoing experiments in our laboratory are exploring this hypothesis. Preliminary data, using Sepharose-coupled tPA pull-down and immunoprecipitation experiments, have failed to detect the three proteins in the same fraction, but more work is required. Furthermore, it is possible that these interactions require additional proteins,⁷⁴ are transient, or require refined conditions for biochemical analyses. A detailed understanding of the complex interactions of plasminogen activators with the ErbB family of receptors and growth factors is also crucial for the de-

velopment of more efficient therapies aimed to block EGFR signaling in cancer patients. In PDA, recent reports point to EGFR blockade as a promising therapy.^{67–69} Our data indicating that EGFR is involved in tPA-induced ERK1/2 signaling suggest that at least part of the inhibition of cell growth resulting from EGFR blockade could be mediated by tPA signaling.

Our results have important implications for the fields of plasminogen system and pancreatic cancer biology. On the one hand, we describe a new tPA non-catalytic function, which should be added to others that have been recently identified. On the other hand, we provide new evidence about the molecular mechanism leading to tPA proliferative effects in pancreatic cancer, which may contribute to the development of new targeting strategies against this aggressive malignancy.

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