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VIEWPOINTS IN DIGESTIVE DISEASES

Tissue Plasminogen Activator Is Required for the Growth, Invasion, and Angiogenesis of Pancreatic Tumor Cells

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Background & Aims: Overexpression of tissue-type plasminogen activator (t-PA) in exocrine pancreatic tumors might be a determinant of the aggressive biological behavior of these tumors. _Methods:_ Endogenous t-PA production was suppressed by antisense oligonucleotides or transcripts in CAPAN-1 and RWP-1 cell lines. Reciprocally, the t-PA non-expressing BxPC-3 and PANC-1 cells were stably transfected to overexpress t-PA. Recombinant t-PA and chemical inhibitors were also used on these cells. Clones were assayed for invasion and growth in vitro and in vivo. _Results:_ In vitro, specific inhibition of t-PA expression or activity significantly inhibited the proliferation of t-PA–producing RWP-1, CAPAN-1, and SK-PC-1 cells. Antisense constructs were used to generate RWP-1 clones stably suppressed for t-PA expression (AS clones). These clones had a significantly reduced invasion and proliferation on plastic and in soft agar. The addition of recombinant t-PA rescued the growth of the AS clones to parental levels and was mitogenic for other independent pancreas cell lines. This effect did not require plasmin activity. In athymic mice, RWP-1 AS clones produced tumors fivefold smaller than control clones. AS tumors contained a significantly reduced number of Ki67-positive nuclei, fewer mitotic cells, and a remarkably reduced angiogenic network. Finally, the generation of tetracycline-repressed t-PA transfectants in PANC-1 cells confirmed the activity of t-PA in invasion and proliferation in vitro and in vivo. _Conclusions:_ t-PA, in addition to its known role in invasion, plays other critical roles in pancreas tumor progression, stimulating cancer cell proliferation and tumor-associated angiogenesis.

Tissue-type plasminogen activator (t-PA) and urokinase (u-PA) are serine proteases that proteolytically activate the zymogen plasminogen to plasmin in vivo.[1] Plasmin plays a prominent role in cancer invasion, with involvement in the degradation of the extracellular matrix,[2] the activation of several prometalloproteases,[3,4] the metastatic process,[5,6] and the activation of latent growth factors such as hepatocyte growth factor and transforming growth factor β[7–9] or the liberation of vascular endothelial growth factor, fibroblast growth factor, platelet-derived growth factor, and insulin growth factor II from the extracellular matrix.[10–12] The role of u-PA and its specific membrane receptor, urokinase receptor, in tumor growth and invasion is well established.[6,13–15] The possible role of t-PA in cancer progression is less well understood. t-PA expression is elevated in patients with familial adenomatous polyposis coli, but it decreases in adenocarcinomas of the colon.[16] In breast carcinomas, patients with high u-PA and low t-PA levels in the tumors have significantly higher relapse rates than those with low u-PA and high t-PA levels.[17] In contrast, in melanoma, neuroblastoma, acute myeloblastic leukemia, and pancreatic cancer, higher levels of t-PA have been associated with invasive and/or metastatic behavior,[18–22] suggesting that t-PA contributes to tumor progression. In pancreatic adenocarcinomas, overexpression of t-PA is specifically observed in cancer cells but not in epithelial cells from the areas of pancreatitis or in primary cultures derived from normal exocrine pancreas.[21] The observation that t-PA expression is strictly associated with the tumor phenotype suggests that if most pancreas tumor cells activate de novo t-PA expression, this protease might be required for processes important in tumor progression. To gain further insight into the role of t-PA in the growth characteristics of pancreas tumors, we have developed in vitro models in which endogenous t-PA

Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle medium; DOTAP, N-[1-(2,3-Dioleoyloxy)-propyl]-N,N,N-trimethylammonium methylsulfate; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; rt-PA, recombinant tissue-type plasminogen activator; t-PA, tissue-type plasminogen activator; u-PA, urokinase.

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levels have been artificially altered. Using several independently derived pancreas tumor cell lines in which expression of t-PA was engineered in vitro, we confirm the role of t-PA in in vitro invasion; most importantly, we report previously undescribed roles for this protease in tumor progression, namely that endogenous t-PA stimulates cell proliferation and that it is required for the development of in vivo tumor-associated neoangiogenesis.

Materials and Methods

Cell Culture and Reagents

Cell lines obtained from the American Type Culture Collection (Rockville, MD) were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO-BRL, Gaithesburg, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO-BRL) at 37°C in an atmosphere of 5% CO_2. Pefabloc/t-PA (2,7-Bis-[4-amidinobenzylidene]-cycloheptanone-[1] dihydrochloride salt) was from Pentapharm (Basel, Switzerland). Matrigel was from Becton Dickinson (Bedford, MA). Recombinant t-PA (rt-PA; Actilyse) was from Boehringer Mannheim (Barcelona, Spain). Agar Noble was from Difco (Detroit, MI). Neutralizing goat antibodies recognizing t-PA or u-PA were from American Diagnostica (Greenwich, CT). Anti–plasminogen activator inhibitor 1 (ESP-1) was kindly provided by Dr. N. Booth (University of Aberdeen, United Kingdom). Mouse monoclonal anti-annexin II antibody was from Transduction Laboratories (Lexington, KY). Rabbit anti-human Ki67 was from DAKO (Glostrup, Denmark). Rat monoclonal antibody to PECAM-1/CD31 was from Pharmingen (San Diego, CA). Peroxidase-coupled anti-goat antibodies were from DAKO. Biotin-labeled mouse anti-goat immunoglobulin was from DAKO, ABC staining kit was from Pierce (Rockford, IL), and peroxidase-coupled streptavidin was from Zymed (San Francisco, CA).

Plasmid Constructions and Transfection

Antisense inhibition of t-PA expression. The complementary DNA (cDNA) encoding the t-PA gene[23] was used to amplify a 440–base pair DNA fragment that included the first codon (upper primer, 5′-TATCTAGACCCACCCCCTGCCTGGAAACTT-3′; lower primer, 5′-ATGGATCCGTGCCCCCGTTGAAACACCTTG-3′) and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) in antisense orientation under the control of the cytomegalovirus promoter. Plasmid DNA was transfected into RWP-1, expressing approximately 5.1 ng of t-PA · 24 hours · μg of total protein by means of cationic liposomes (Lipofectamine; GIBCO-BRL) and stable transfectants selected in medium with 10% FBS containing G418 (600 μg/mL) (GIBCO-BRL). Overall, 38 clones were selected, slow-growing clones were discarded, and the remaining 33 clones were analyzed by enzyme-linked immunosorbent assay and Western blotting to determine the t-PA protein levels. Five clones showed a reduction of more than 90% of secreted protein (<0.3 ng of t-PA · 24 h · μg of total protein), 11 had a decrease in secreted t-PA that ranged between 70% and 90%, and 17 produced t-PA levels that were not significantly different from parental cells. Also, specific inhibition of t-PA expression was achieved by transfecting CAPAN-1 cells with antisense oligonucleotides directed to the 5′ region of the coding sequence of t-PA (t-PA-AS, 5′-CCCTCTCTTCATTGCATCCAT-3′). Oligonucleotides with phosphorothioate modification at the 4 external bases (TIB MOLBIOMOL, Berlin, Germany) were transfected in combination with DOTAP (Boehringer-Mannheim, Mannheim, Germany) according to described protocols.[24] The optimal ratio concentration of the transfection mix was determined to be 2 μmol/L of oligonucleotides and 8 μmol/L of DOTAP in DMEM medium with 1% FBS. Inhibition of t-PA expression was confirmed by Western blotting of cells exposed to transfection mix for 72 hours.

Overexpression of t-PA in nonexpressing cell lines. The full-length t-PA cDNA[23] inserted into pcDNA3 in sense orientation was transfected into BxPC-3 cells as previously described and stable clones selected in G418-containing medium (400 μg/mL). Tetracycline-regulated overexpression of t-PA was obtained in PANC-1 cells by first transfecting the pTet-Off plasmid (Clontech, Palo Alto, CA) and selecting transfectants in medium containing G418 (400 μg/mL). A second transfection with pTRE-tPA and pTK-hygromycin plasmids was then performed in a chosen Tet-Off transfectant, and a second selection medium containing hygromycin (100 μg/mL) and G418 was applied for 3 weeks. Three of 45 clones were further selected for showing, by Western blotting, high induction of t-PA expression in the absence of tetracycline and complete shutdown of t-PA expression in the presence of tetracycline. These clones were routinely maintained in medium containing tetracycline (2 μg/mL; Sigma Chemical Co., St. Louis, MO), G418, and hygromycin.

RNA Analysis

Total RNA was obtained by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure.[25] Northern blot analysis of total RNA (15 μg) was performed according to standard procedures.[20] RNA quality was evaluated by ethidium bromide staining, and filters were normalized by hybridization with a probe for glyceraldehyde-3-phosphate dehydrogenase. Reverse transcriptions were performed with 2 μg of total RNA using oligo(dT) and M-MLVRT (Promega, Madison, WI). Amplification reactions were performed with T7 and SP6 primers.

Western Blotting

Cells were lysed with Laemmli sample buffer (50 mmol/L Tris-HCl, pH 6.8, 2% [wt/vol] sodium dodecyl sulfate, 10% [vol/vol] glycerol) heated at 85°C and centrifuged at 10,000*g*. Protein (40 μg) gel electrophoresis and transfer to nitrocellulose were performed as described.[21] Filters were blocked with 3% bovine serum albumin in phosphate-buffered saline/0.1% Tween 20 and incubated with primary antibody,

and antigen was detected by horseradish peroxidase–conjugated secondary antibody. After washing, reactivity was developed with a chemiluminescent substrate (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England). For sequential Western blotting, membranes were stripped at 50°C according to the manufacturer's instructions.

Cell Proliferation Assays

Cells (1.5×10^4/well) were plated in 24-well plates in complete medium, and replica plates were switched to 1% FBS medium after 24 hours. Medium was replaced every other day. A total of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) was added for 3 hours at 37°C to determine the percentage of viable cells. After solubilization of cells in acidic isopropanol (0.1N HCl), the absorbance at 549–630 nm was determined. Proliferation was also determined by total protein quantification using the method of Bradford (Bio-Rad, Richmond, VA) and by cell counting after trypsinization. The treatment of cells with Pefabloc/t-PA (10 or 30 μmol/L) for the indicated lengths of time was determined not to be toxic, as shown by the lack of release of lactate dehydrogenase (not shown). To determine the rate of DNA synthesis, cells grown in 0.5% FBS for 72 hours were exposed to factors for 22 hours in the presence of 0.5 μCi [^{3}H]thymidine/well (Amersham Pharmacia Biotech). Labeled DNA was precipitated with 15% (wt/vol) trichloroacetic acid.[26] The effect of the addition of mitogen was expressed as a percentage of the incorporation of [^{3}H]thymidine in cells grown at 0.5% FBS.

In Vitro Invasion Assay

The invasive potential of cultured tumor cells was tested using Matrigel-coated Transwell filters (Costar, Cambridge, MA). Quantitative determinations were obtained using overnight [^{3}H]thymidine-labeled cells (2 μCi · 10^5 cells · mL) according to the procedure previously described.[21] Results are expressed as the percentage of labeled cells on the bottom surface of the filter with respect to the total represented by the cells present in the upper and lower chambers.

Soft-Agar Growth Assay

Anchorage-independent growth was evaluated as described.[27] Cells were plated at different dilutions (1, 4, or 20 × 10^3 cells/mL in complete DMEM containing 0.3% agar) in 6-well plates. Medium was added (100 μL/well) twice a week to maintain constant humidity. After 3 weeks, colonies ≥0.2 mm in diameter containing approximately 30–50 cells were counted by staining with MTT (0.5 mg/mL) for 3 hours at 37°C.

Tumorigenic Assay in Nude Mice

RWP-1 clones were grown in medium without G418 for 6 days, trypsinized when 70% confluence was reached, washed, and resuspended in sterile phosphate-buffered saline. These cells as well as SK-PC-1, CAPAN-1, BxPC-3, and PANC-1 cells (2×10^6) were inoculated in the subcutis of 5-week-old athymic female BALB/c mice (Criffa, Barcelona, Spain). Two injections per mouse were performed, one on each thigh of the animal, and 3 mice per clone were used. Tumor volume was measured externally with a caliper once a week and calculated by the formula of an ovoid in which L equals midaxis length and W equals midaxis width: Tumor Volume $= 4/3\pi \times L/2\ (W/2)^2$. After 15 weeks, mice were killed by cervical dislocation, and the site of injection as well as the liver, lungs, and lymph nodes were examined. Tissues were snap-frozen in liquid nitrogen using OCT or formalin-fixed, embedded in paraffin, and stained with H&E.

To decrease the latency of tumor formation, PANC-1 Tet-Off clone 21 (4×10^6 cells) grown in the presence or absence of tetracycline were inoculated into nude mice after resuspension in 200 μL growth factor–depleted Matrigel (Becton Dickinson). When indicated, mice received tetracycline in the drinking water (2 mg/mL) with a change every other day. Tumor mass was monitored every week and tumor volume calculated as previously described.

Immunohistochemical Methods

Ki67 antigen was detected by the indirect immunoperoxidase assay in 5-μm paraffin-embedded sections treated with heat in citrate buffer for antigen retrieval. Endogenous peroxidase activity was quenched with 3% H_2O_2; sections were incubated with primary antibody, washed, and thereafter incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin (EnVision; DAKO). Subsequently, sections were washed and reactions were developed with diaminobenzidine, followed by counterstaining with hematoxylin. Quantitative determinations for Ki67-positive nuclei were performed by 2 independent investigators by counting the number of positive nuclei per number of total cells per high-power field (magnification 400×). A minimum of 700 total nuclei was counted for each sample. PECAM-1/CD31 antigen was detected on 10-μm cryostat sections, and stained cells and microvessels were counted according to previously described methods.[28] Areas of highest vascularization were chosen at low magnification (100×) and microvessel counting performed at 200× on 3 chosen fields. Three consecutive sections were used for quantitative determinations. The microvessel score was the average of the vessel counts obtained in the 3 sections. Microvessels adjacent to normal tissue were excluded from the appraisal. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting. We separately assessed the endothelial cell score by counting single endothelial cells in the same fields in which microvessel counting was performed. In all assays, matched isotype control antibodies were used and found to be unreactive in all cases.

Statistics

Results are expressed as mean ± SEM, and the Student t test was used for statistical analysis. $P < 0.05$ was taken as level of significance.

Results

Endogenously Produced t-PA Is Necessary for Proliferation of Pancreas Cancer Cells in Low Serum Medium

t-PA stimulates the proliferation of untransformed cells in vitro.[29,30] To study the function of t-PA in pancreatic adenocarcinomas, we have inhibited its expression by transfecting CAPAN-1 and SK-PC-1 cells, which overexpress the gene,[21] with oligonucleotides complementary to the 5′ region of the t-PA messenger RNA (mRNA) that encompasses the translational start site (t-PA-AS). As shown by Western blotting (Figure 1*A*), the oligonucleotide t-PA-AS at 2 μmol/L caused an inhibition of t-PA protein in CAPAN-1 cells of more than 90%, as determined by densitometric analysis with normalization to cells treated with oligonucleotide t-PA-sense. No decrease of t-PA expression was observed in cells transfected with t-PA-sense or in sham-transfected cells (Figure 1*A*) or with oligonucleotides complementary to several other regions of the gene (not shown).

Down-regulation of t-PA expression was stable for at least 72 hours after transfection of t-PA-AS oligonucleotides. To determine if the reduced t-PA production in CAPAN-1 cells affects their growth, cell proliferation was monitored for 6 days by MTT. Because the presence of serum may mask the activities of growth factors, cells were grown in low (1%) serum. Control cells not transfected, sham transfected, or transfected with the t-PA-sense oligonucleotide grew at the same rate and reached confluency at day 6 (Figure 1*A* and data not shown). In contrast, t-PA-AS–treated cells had a significant decrease in their proliferative capacity at day 6 of growth. The average from 3 independent experiments showed a decrease in proliferation of 53% compared with sham-transfected cells.

To determine if active t-PA is necessary for proliferation, we treated cells with Pefabloc/t-PA, a synthetic derivative of benzamidine that, at the concentration used, inhibits t-PA but not u-PA or plasmin activities.[21,31] CAPAN-1 and SK-PC-1, expressing high levels of t-PA,[21] and RWP-1, expressing moderately high levels of t-PA, were grown in 1% FBS without or with the addition of Pefabloc/t-PA. All cell lines treated with the inhibitor showed a dose-dependent decrease of proliferation as compared with untreated cells, with inhibition of proliferation of more than 90% at 30 μmol/L (Figure 1*B* and data not shown). The effect of Pefabloc/t-PA was not observed when cells were grown in the presence of 10% FBS (not shown). Thus, proteolitically active t-PA is required for proliferation of pancreas cancer cells in vitro. In addition to these experiments, the t-PA–expressing cell lines SK-PC-1, CAPAN-1, and RWP-1 (which produce 20, 18, and 5 ng of t-PA · 24 h · μg total protein, respectively) as well as BxPC-3 and PANC-1 cell lines, which do not express t-PA,[21] were injected in the subcutis of nude mice, and growth was monitored every week (Figure 1*C*). At 6 weeks after injection, only the high t-PA expressor cells SK-PC-1 and CAPAN-1 formed tumors. At 14 weeks, tumors were observed only in the t-PA–producing SK-PC-1, CAPAN-1, and RWP-1 cells. BxPC-3 and PANC-1 cells showed a latency of tumor formation longer than 4 months. These results indicate that there is a correlation between the faster in vivo growth rate of t-PA–producing cancer cells and their levels of t-PA expression.

Stable Inhibition of t-PA Expression in RWP-1 Cells by Antisense Transcripts Decreases Invasion and Cell Proliferation Abilities

To confirm the growth-promoting effect of t-PA on pancreatic adenocarcinomas, we have stably inhibited its expression by transfecting RWP-1 pancreas tumor cells with a construct expressing t-PA antisense transcripts under the control of a constitutive promoter (AS clones). Northern and Western blotting of 4 transfectants showed a marked decrease in t-PA levels (AS-2, AS-3, AS-7, AS-29) compared with parental cells and control clones transfected with empty vector (C-1, C-13) (Figure 2*A* and *B*).

The levels of t-PA transcripts in the AS clones were reduced by more than 90% with respect to control clones, as indicated by densitometric analysis with normalization to glyceraldehyde-3-phosphate dehydrogenase signal. The suppression of t-PA expression was dependent on the expression of antisense transcripts, as determined by reverse-transcription polymerase chain reaction with primers specific for the exogenous construct and transcript (not shown). No major variations in the levels of u-PA mRNA were observed in the different clones analyzed. A parallel significant reduction of t-PA protein levels was detected in all AS clones. No major variations in the levels of annexin II, the putative membrane receptor for t-PA in endothelial cells,[32] and plasminogen activator inhibitor 1, the physiologic inhibitor of t-PA, were detected in the AS clones with respect to control clones.

We have previously shown that t-PA contributes significantly to the in vitro invasiveness of SK-PC-1 pancreas cancer cells.[21] To confirm this activity for t-PA in RWP-1 cells, 2 control and 3 AS clones were tested for their capacity to invade through a reconstituted basal membrane (Matrigel) in vitro. After 72 hours of incubation, the invasive abilities of clones AS-2, AS-3, and AS-29 were reduced by 51%, 62%, and 42%, respec-

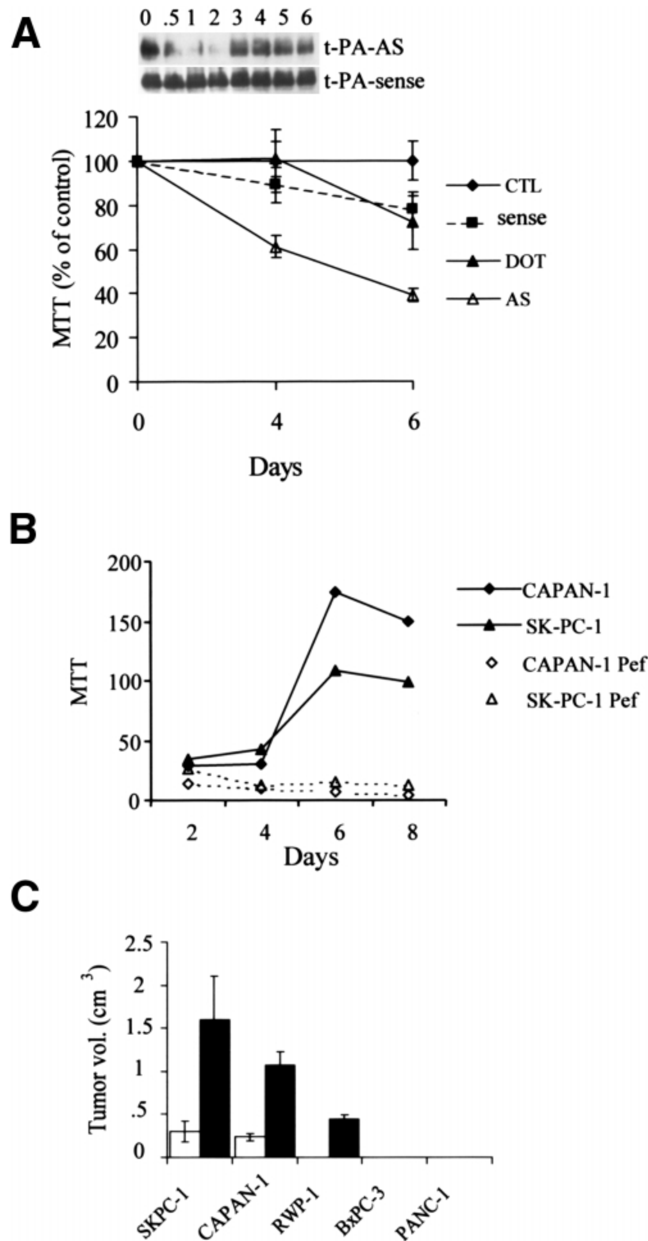

Figure 1. Endogenous t-PA is necessary for the proliferation of pancreas tumor cells in low-serum medium. (*A*) *Upper panel*: t-PA expression (Western blotting) of CAPAN-1 cells treated for 72 hours with different concentrations (μmol/L) of the antisense oligonucleotide to t-PA (t-PA-AS) or control oligonucleotide (t-PA-sense) and a fixed concentration of DOTAP (8 μmol/L). *Lower panel*: CAPAN-1 cells not transfected (CTL), sham-transfected (DOT, DOTAP only), or treated with 2 μmol/L t-PA-AS or with 2 μmol/L t-PA-sense plus DOTAP were monitored for proliferation by MTT every 2 days with a change of transfection mix at 72 hours. Results obtained at days 4 and 6 are shown. One representative experiment is shown of 3 performed in quintuplicate replicas. (*B*) CAPAN-1 and SK-PC-1 tumor cells overexpressing t-PA were plated sparsely (1.5×10^4/well) in 24-well plates in DMEM plus 10% FBS and, after 24 hours (day 1), were switched to 1% FBS without or with Pefabloc/t-PA (30 μmol/L, Pef). Cell proliferation was monitored by MTT incorporation for 8 days. Each point represents the mean of triplicate determinations. (*C*) In vivo growth of pancreas tumor cell lines. SK-PC-1, CAPAN-1, RWP-1, BxPC-3, and PANC-1 cells were grafted in the subcutis of athymic mice. Tumor growth was monitored every week and tumor volume calculated as described in Materials and Methods. The results at 6 weeks (□) and 14 weeks (■) after inoculation are shown.

tively, with respect to the invasion of parental and C-13 control cells ($P = 0.02$ comparing the group AS with the group control) (Figure 2*C*). Because all clones produce u-PA and express similar levels of plasminogen activator inhibitor 1, we concluded that the diminished capacity of invasion of the AS clones is a consequence of their decreased t-PA production.

We next analyzed whether endogenous t-PA production is required for the proliferation of RWP-1 cells. The control clones C-1 and C-13 grew at the same rate as the parental untransfected cells in the presence of either 10% or 1% FBS (Figure 2*D*). In contrast, AS clones were not able to proliferate in 1% serum, although no changes from control clones were observed in the presence of 10% FBS. Similar results were obtained when cell proliferation was determined by cell counting or total protein determination (not shown). Furthermore, the addition of rt-PA (50 ng/mL) stimulated DNA synthesis in these cells 2–2.8-fold with respect to untreated control cells (Figure 2*E*). These results show that endogenous t-PA is necessary for the proliferation of RWP-1 cells.

t-PA Is Required for Anchorage-Independent Growth of RWP-1 Cells

We analyzed the capacity of RWP-1 parental cells and AS clones to grow in anchorage-independent conditions. Clones AS-2, AS-3, AS-7, and AS-29 showed a significant reduction in their capacity to form colonies in soft agar when compared with control clones and parental cells (Figure 3; $P < 0.02$, $P < 0.004$, $P < 0.0001$, and $P < 0.01$, respectively, when compared with C-1). rt-PA was able to restore the ability of the AS clones to grow in agar but did not further increase the number of colonies formed by control clones (not shown).

In a reciprocal manner, the addition of the inhibitor Pefabloc/t-PA to the agar-containing medium resulted in a significant inhibition of colony formation in control clone C-13 ($P = 0.024$). Thus, active t-PA is required for full growth of RWP-1 cells in anchorage-independent conditions, suggesting a role for t-PA in the tumorigenic ability of these cells.[33]

Endogenous t-PA Is Required for RWP-1 Cells to Grow and to Activate Angiogenesis in Nude Mice

The in vivo tumorigenic potential of RWP-1 AS clones and control cells was tested in athymic mice. Tumors arising from clones AS-2, AS-3, AS-7, and AS-29 consistently showed a significantly slower growth rate with respect to tumors arising from control clones (Figure 4). At 15 weeks, the AS clones grew fivefold less with respect to the control group (Figure 4*A*). Kinetic analyses showed that at any time after cell xenograft,

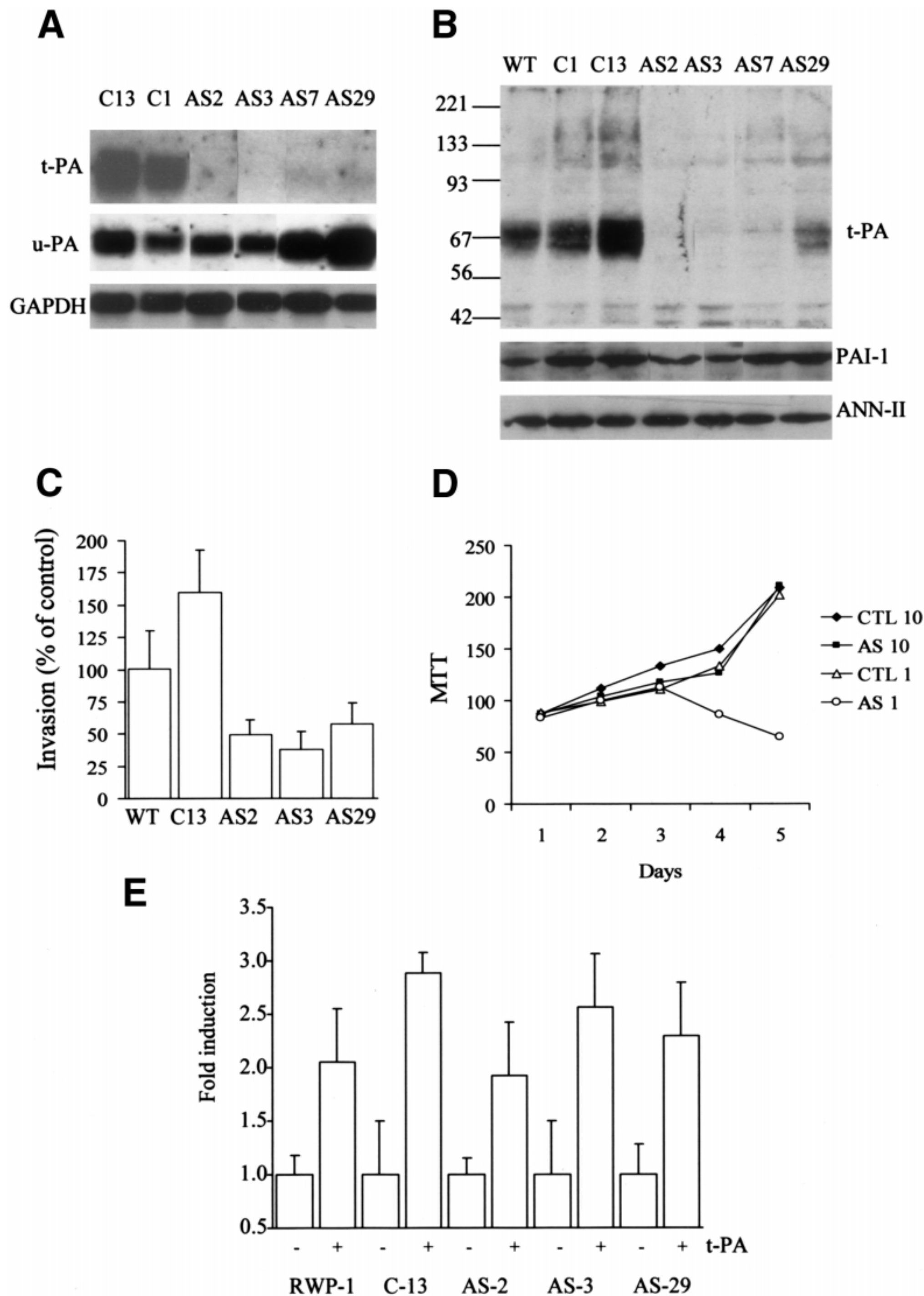

Figure 2. Stable suppression of t-PA expression in RWP-1 cells by antisense t-PA transcripts. Effect on cell invasion and proliferation in vitro. (*A*) Northern blotting of total RNA (15 μg) extracted from control C-13, C-1, AS-2, AS-3, AS-7, and AS-29 RWP-1 transfectants. Membranes were sequentially hybridized to a t-PA probe and a u-PA probe. The blots were normalized for equal loading by hybridization with a glyceraldehyde-3-phosphate dehydrogenase probe. (*B*) Sequential Western blotting analysis of t-PA, plasminogen activator inhibitor 1 (PAI-1), and annexin II (ANN-II) expression. Equal amounts of total cell lysates (40 μg) from the indicated transfectants were loaded, RWP-1 parental cells (WT). (*C*) In vitro cell invasion assay. Cells were cultured on Matrigel-coated Transwell filters for 72 hours. Quantitative determinations of cells invading the bottom chamber were performed with [^{3}H]thymidine-labeled cells (see Materials and Methods). (*D*) Proliferation was determined by MTT incorporation at the indicated days. Cells were grown in 10% FBS (CTL 10 represents the average of results from C-1, C-13, and parental RWP-1 cells; AS 10 represents the average of results from AS-2, AS-3, and AS-29 clones) or 1% FBS (CTL 1 and AS 1). (*E*) Thymidine incorporation assay. Cells were cultured in 24-well plates in DMEM plus 10% FBS. After cell attachment, media was switched to DMEM plus 0.5% FBS. After 2 days, cells were treated with rt-PA, and [^{3}H]thymidine incorporation into DNA was determined after 22 hours. All experiments were performed in triplicate samples and repeated twice. The *error bars* represent the SEM. For statistical analysis, see the text.

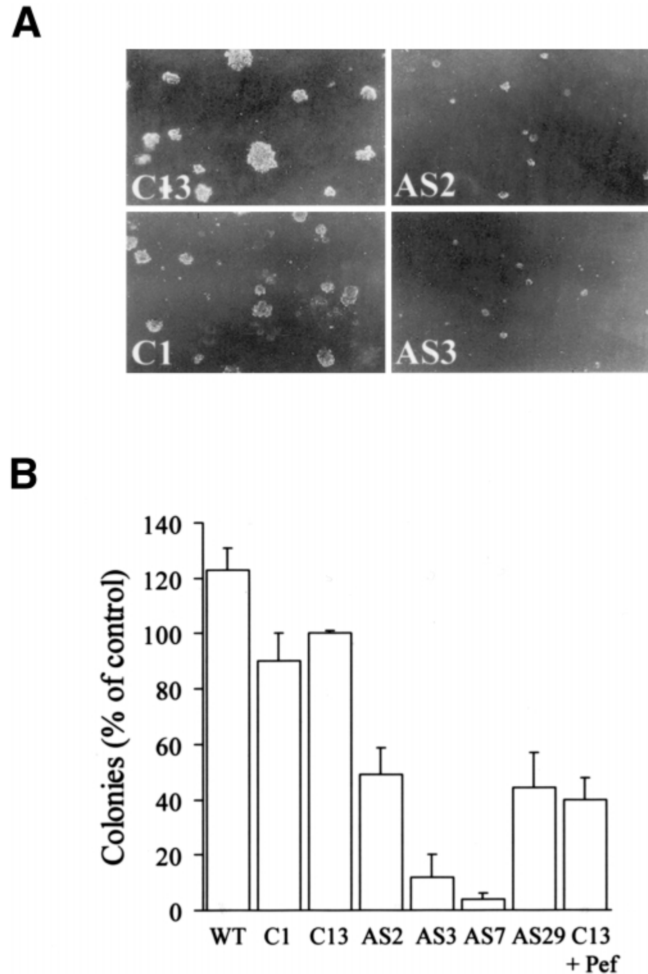

Figure 3. t-PA permits optimal growth of RWP-1 clones in soft agar. Cells (4000/well) were seeded in triplicate 6-well plates in a 0.3% agar solution as described in Materials and Methods. After 3 weeks, colonies were counted. (*A*) Representative wells for control transfected (*left panels*: C-1 and C-13) and antisense transfected clones (*right panels*: AS-2 and AS-3) are shown (original magnification 100×). (*B*) Quantitative evaluation of MTT-stained colonies. Where indicated, the t-PA specific inhibitor Pefabloc/t-PA (Pef, 10 μmol/L) was added to C-13 cells. The *error bars* represent the SEM. Three independent experiments were performed in triplicate. Interexperiment variation of 10% or less was observed. For statistical analysis, see the text.

tumor volumes were significantly decreased for AS clones compared with control clones (at week 7, $P < 0.005$; at week 15, $P < 0.001$).

Tumors derived from control clones and parental cells contained many atypical cells with large pleomorphic and hyperchromatic nuclei, forming glandular-like structures with a poorly differentiated phenotype (Figure 4*D* and *F*). In contrast, tumors derived from the AS clones appeared more differentiated (moderately differentiated) with larger glandular structures and smaller, more uniform cells with fewer pleomorphic nuclei (Figure 4*E* and *G*). The desmoplastic reaction seemed more abundant in these tumors compared with tumors derived from control cells. No differences were observed in the local microinvasion, and metastases to distant organs were not observed by macroscopic analysis in mice killed 15 weeks after tumor cell inoculation.

To assess the proliferative rates of these xenotransplants, the expression of the proliferation-associated antigen Ki67 in nuclei was studied by immunohistochemistry. The proportion of stained nuclei in tumors derived from AS-3 and AS-29 clones (16% ± 5.7% and 15% ± 1.9%) was significantly lower compared with parental RWP-1 and C-13 cells (22% ± 3.4% and 26% ± 3.0%, respectively) ($P < 0.001$ comparing the controls with the AS group) (Figure 4*B*). Moreover, a significantly higher proportion of mitosis was observed in control tumors compared with antisense tumors ($P < 0.0001$). These results confirm that endogenous t-PA production stimulates proliferation of RWP-1 cells in vivo.

Previous results from others have shown that t-PA may regulate the angiogenic process stimulating endothelial tube formation in vitro.[34–36] To identify possible differences in the angiogenesis of the tumors formed by RWP-1 AS and control clones, we studied the expression pattern of the endothelial cell marker PECAM-1/CD31. Tumors derived from parental cells or control clones showed a well-developed angiogenic network, with long and thick vessels predominating over occasional single endothelial cells (Figure 4*H* and *J*). In striking contrast, tumors derived from RWP-1 AS clones contained mainly single endothelial cells, with only occasional small and thin vessels (Figure 4*I* and *K*). To quantitate this difference, we counted the number of microvessels and endothelial cells and the total number of stained structures (vessels plus single cells) per field (Figure 4*C*). Tumors derived from control cells expressing t-PA contained approximately the same number of vessels and isolated cells, whereas tumors derived from AS clones contained a few vessels but numerous isolated endothelial cells. The total number of structures (microvessel score plus endothelial cell score) was not significantly different when comparing control and AS-derived tumors. These results underscore the importance of the t-PA produced by pancreatic cells for the correct development and maturation of the tumor-associated angiogenic network.

t-PA Induces Proliferation in BxPC-3 and PANC-1 Cells Independently of Plasmin Activation

To confirm that the t-PA level of expression modulates cell proliferation, we next performed reciprocal experiments in which the cell lines BxPC-3 and

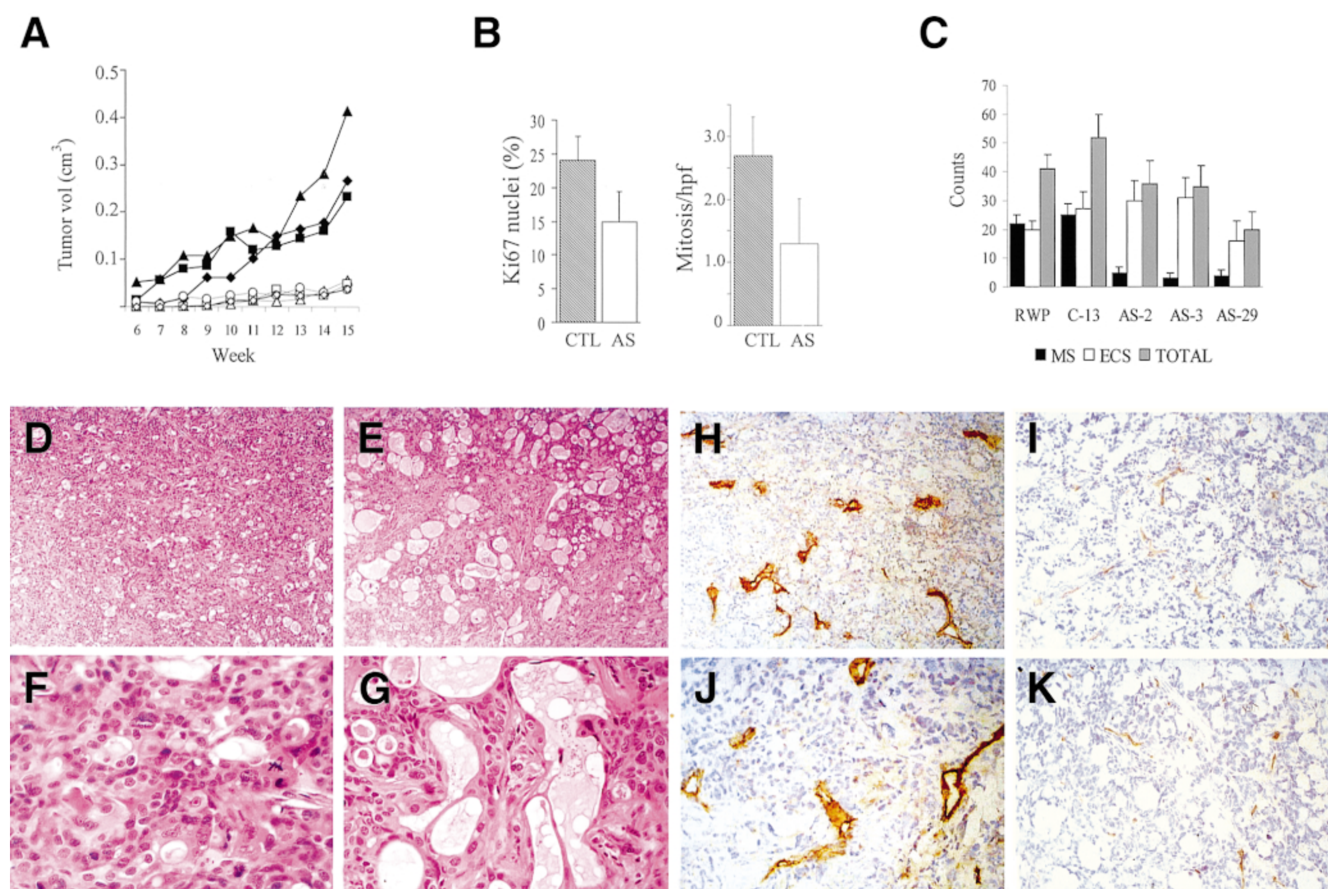

Figure 4. Suppression of endogenous t-PA expression in RWP-1 cells inhibits tumor growth and angiogenesis. (*A*) Growth of RWP-1 cells grafted in nude mice. Control (■, C-1; ▲, C-13; ◆, RWP-1 parental cells) and AS clones (○, AS-2; □, AS-3; ◇, AS-7; △, AS-29) were injected subcutaneously into athymic mice (3 mice per clone). Each point is the mean of 6 tumor measurements (tumor volume). (*B*) *Left panel*: Expression of the proliferation-associated antigen Ki67 determined by immunohistochemistry in tumor nuclei derived from parental and control C-13 cells (▨) and in AS-3 and AS-29 clones (□). Results are expressed as the percentage of positive nuclei per total nuclei counted. *Right panel*: The mitotic index of tumors, as determined by counting mitotic figures per high-power field (original magnification 400×) of tumors derived from parental and control C-13 cells (▨) and AS-3 and AS-29 (□) clones. Twenty fields were counted per tumor. (*C*) Quantification of neoangiogenesis, performed as described in Materials and Methods. The following were assessed: microvessel score (MS), as the average of the vessel counts obtained in the 3 sections; endothelial cell score (ECS), as the single endothelial cells; and total number of stained structures, as vessels plus isolated endothelial cells per field. The results are the mean of independent determinations obtained by 2 investigators. The *error bars* represent the SEM. For statistical analysis, see the text. (*D–G*) Histologic analysis of nude mice tumors derived from RWP-1 cells. Sections (5 μm) of nude mice tumors derived from (*D* and *F*) control C-13 and (*E* and *G*) AS-3 clones were analyzed by H&E staining of paraffin-embedded sections. (*H–K*) Tumor neoangiogenesis was estimated by immunohistochemical analysis with antibodies to PECAM-1/CD31 on cryostatic sections of tumors derived from (*H*) control C-13 cells, (*J*) parental RWP-1 cells, (*I*) AS-3 clone, and (*K*) AS-2 clone. Preparations were counterstained with hematoxylin. (Original magnification: *D*, *E*, *H*, and *I*, 100×; *F* and *G*, 400×; *J* and *K*, 200×).

PANC-1, that do not express t-PA, were engineered to overexpress t-PA (Figures 5 and 6). Consistent with the previously described observations, BxPC-3 clones t-PA10 and t-PA12, expressing high levels of t-PA from a stably transfected construct (Figure 5*A*), showed a significantly increased proliferative capacity compared with parental cells or control clones when analyzed in low serum (Figure 5*B*) ($P < 0.001$ compared with control parental and control transfected cells). This specific increase returned to control values when t-PA10 and t-PA12 cells were treated with Pefabloc/t-PA.

Treatment of control clones or parental cells with the same concentration of inhibitor did not decrease cell proliferation, indicating both its lack of toxicity and its specificity for t-PA. rt-PA also stimulated proliferation of parental cells.

PANC-1 cells also responded to rt-PA with proliferation (Figure 5*C* and *D*). This mitogenic activity was specifically associated with t-PA because Pefabloc/t-PA but not plasmin inhibitors, such as aprotinin and ε-amino caproic acid, were able to block the t-PA–stimulated proliferation. In addition, plasmin did not stimulate DNA synthesis in these conditions (Figure 5*D*). These observations further confirm an essential role of t-PA for the growth of pancreas tumor cells of independent derivation.

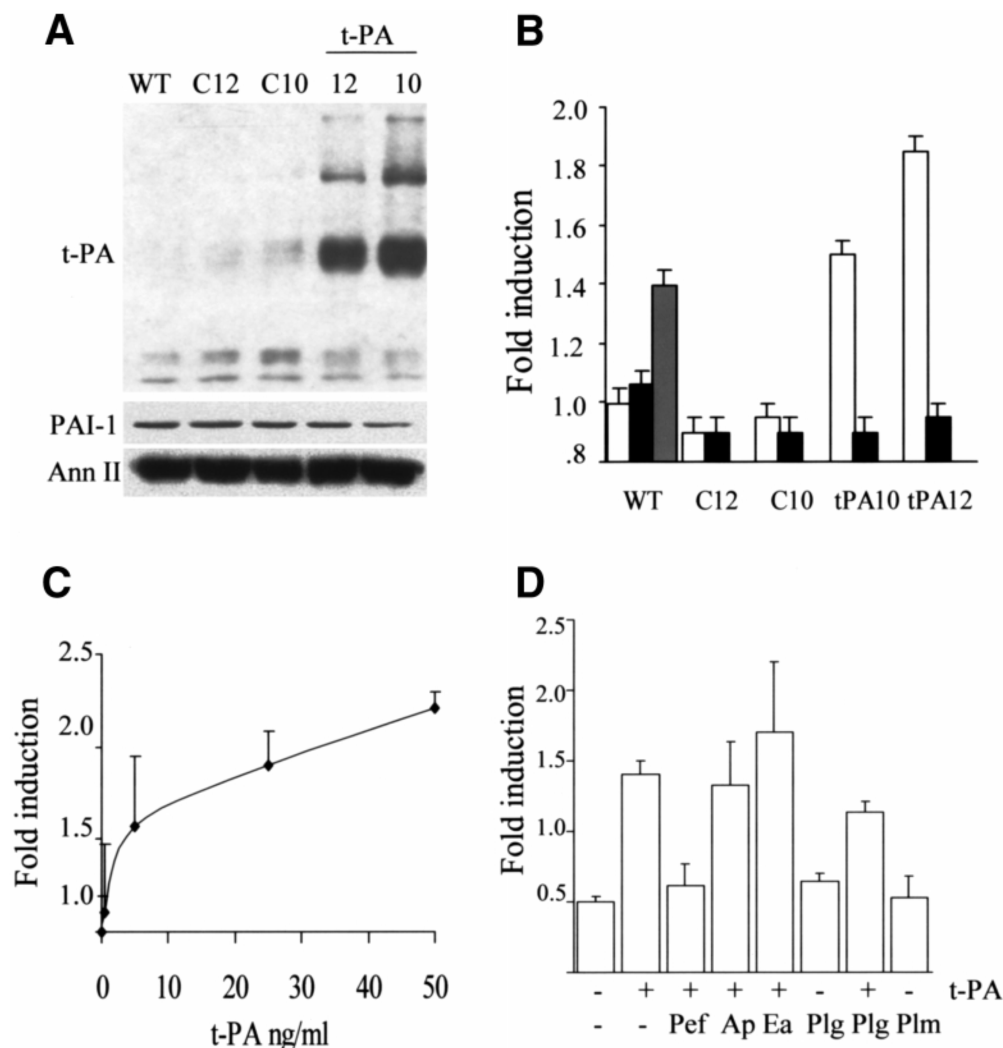

Figure 5. t-PA stimulates proliferation of the t-PA nonexpressing BxPC-3 and PANC-1 cells independently of plasmin. (*A*) Immunoblotting analysis of BxPC-3 parental cells (WT), control clones C10 and C12 transfected with empty vector, and t-PA–transfected cells (t-PA10, t-PA12) was performed as described. (*B*) Cell proliferation was monitored for 6 days in 1% FBS by MTT. The results shown are at day 6 for growth of control and t-PA–expressing clones (□), replica plates grown in the presence of Pefabloc/t-PA (10 μmol/L) (■), or in the presence of rt-PA (50 ng/mL) (▩). The mean optical density values (±SEM) of triplicate determinations were analyzed with respect to control values (WT). A representative experiment is shown from 3 performed with similar results. (*C*) [^{3}H]thymidine incorporation assay of PANC-1 cells treated with rt-PA. Dose-response curve. (*D*) [^{3}H]thymidine incorporation assay with PANC-1 cells treated with t-PA (25 ng/mL), Pefabloc/t-PA (Pef, 30 μmol/L), aprotinin (Ap, 100 μmol/L), ϵ-aminocaproic acid (Ea, 10 mmol/L), plasminogen (Plg, 2 μg/mL), or plasmin (Plm, 0.1 μmol/L). Plasminogen and plasmin inhibitors concentrations used here were determined to be effective in pancreas tumor cell invasion.[21,46] Each point represents the mean of triplicate measurements, and the *error bars* represent the SEM.

Inducible t-PA Overexpression in PANC-1 Cells Increases Invasion and Stimulates Proliferation and Tumor Growth In Vivo

To generate a system for the inducible expression of t-PA and to avoid possible effects of clonal variability, we introduced the t-PA cDNA into PANC-1 cells by the tetracycline-regulated expression system (Tet-Off). After 2 consecutive transfections, we selected clones P21, P37, and P39, expressing high levels of t-PA without tetracycline, whereas no t-PA could be detected in the presence of the antibiotic (Figure 6*A*).

As expected, in the absence of tetracycline, these clones had a significantly increased growth capacity (2.5-fold increase for P21 cells) as monitored by MTT assays, and this correlated with the levels of t-PA expression (Figure 6*C*). As observed previously, no differences in the rate of proliferation were detected in the presence of 10% FBS (not shown). In vitro invasion was analyzed with PANC-1 P21 clones grown on Matrigel-coated Transwell filters with or without tetracycline (Figure 6*B*). P21 cells producing t-PA (no tetracycline) had a significantly increased capacity of invasion (38% more) with respect to

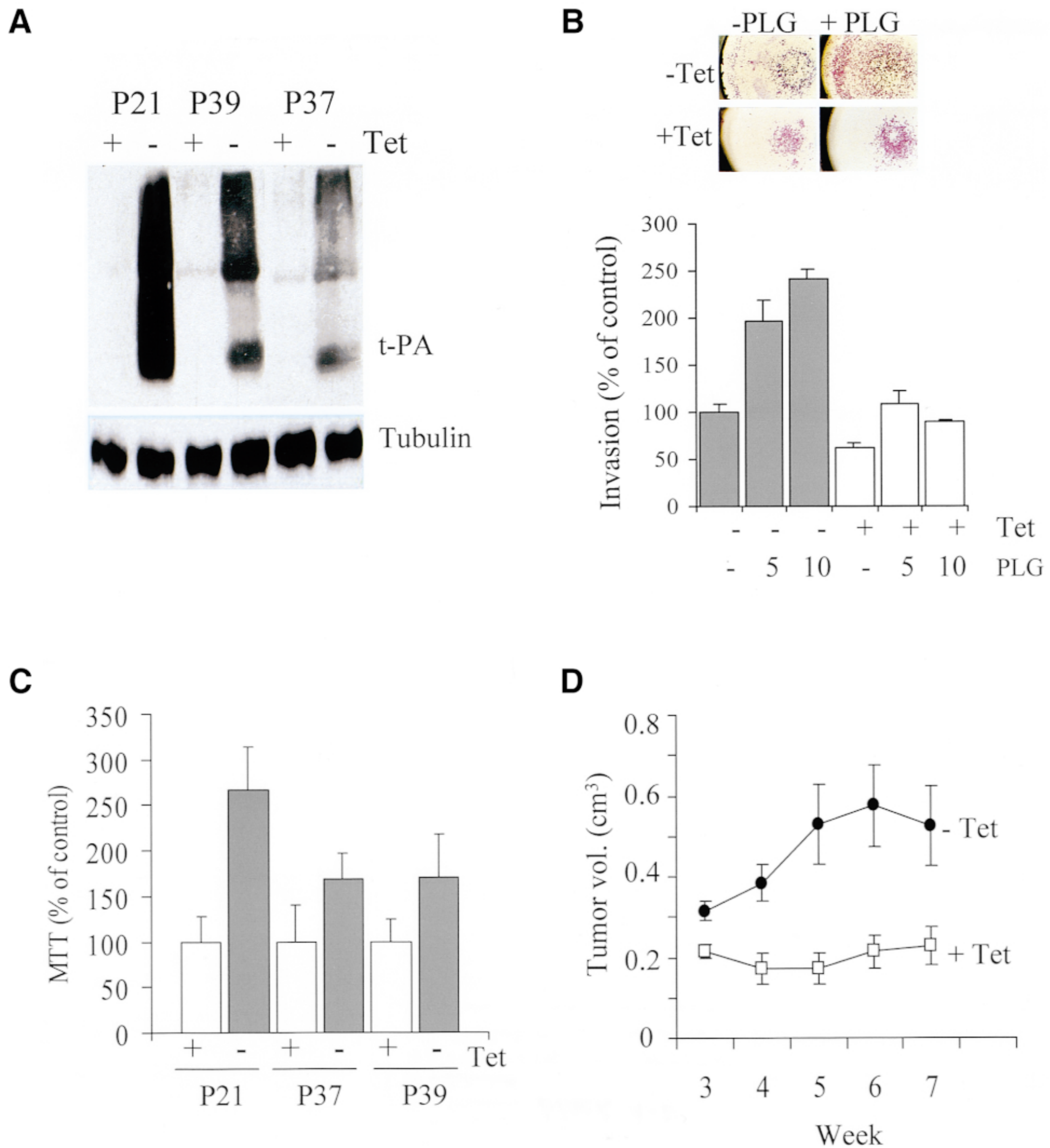

Figure 6. Conditional expression of endogenous t-PA in PANC-1 cells increases invasion and stimulates growth in vitro and in vivo. (*A*) Immunoblotting analysis of Tet-Off PANC-1 clones. Clones P21, P37, and P39 were grown without or with tetracycline (2 μg/mL) in complete medium for 3 days. Immunoblotting analysis was performed with anti–t-PA antibody as previously described. After stripping, the membrane was normalized with anti-tubulin antibody. (*B*) In vitro cell invasion assay. P21 transfectants were cultured on Matrigel-coated Transwell filters for 72 hours. Quantitative determinations of cells invading the bottom chamber (*inset*, cells stained with crystal violet) were performed as described in Materials and Methods. PLG, plasminogen (μg/mL); tet, tetracycline. (*C*) Cell proliferation was monitored for 6 days in 10% or 1% FBS by MTT in the absence or presence of tetracycline. The results shown are for clones P21, P37, and P39 at day 6 of growth in 1% FBS; cells producing t-PA (without tetracycline, ■) or cells without t-PA (plus tetracycline 2 μg/mL, □). (*D*) PANC-1 cells growth in nude mice. P21 transfectants grown in the absence (●) or presence (□) of tetracycline were injected (4×10^6 cells) subcutaneously into athymic mice as described in Materials and Methods. Where indicated, mice were treated with tetracycline in the drinking water (2 mg/mL). Tumor growth (volume) was measured every week starting 3 weeks after injection.

the same cells with tetracycline. Addition of plasminogen greatly stimulated invasion of cells in the t-PA–producing state although only poorly stimulated invasion of cells with no t-PA, indicating that plasmin, generated via t-PA, is a major activator of invasion in these cells.

Finally, to confirm the role of t-PA in the ability of pancreas cells to form tumors in vivo, PANC-1 P21 clones were grown in athymic mice and tumor growth was monitored for 7 weeks. As expected, tumors expressing t-PA in mice that received no tetracycline showed a significantly faster growth with respect to tumors arising in mice treated with the antibiotic (Figure *6D*). At week 7 after inoculation, P21 tumors without tetracycline had grown 2.3-fold more than control P21 tumors, confirming our previous findings.

Discussion

There is much evidence that the plasminogen activator system plays a major role in tumor cell invasion. The results shown here indicate that t-PA, in addition to being required for a full invasive phenotype of cancer cells, is necessary for the growth of different pancreas tumor cell lines in vitro and in vivo and is required for tumor-associated neoangiogenesis in vivo.

The mitogenic effect of t-PA on pancreas tumor cells is shown here in vitro and in vivo by a number of complementary experimental approaches performed on cells with different genetic backgrounds. In 1 set of experiments, the inhibition of expression of the endogenous t-PA by antisense oligonucleotides in CAPAN-1 cells or by an antisense expression vector stably integrated into RWP-1 cells resulted in a marked reduction of the proliferation capacity on plastic and in soft agar. These capacities were rescued to parental cell values in RWP-1 AS clones by adding exogenous t-PA back into the culture medium, thus arguing that suppression of endogenous t-PA expression is the only responsible factor for the compromised growth of the AS clones. Consistent with these observations, blocking t-PA proteolytic activity with the specific inhibitor Pefabloc/t-PA, used at concentrations that do not inactivate u-PA or plasmin,[31] also inhibited proliferation of several pancreas tumor cell lines expressing t-PA. In reciprocal experiments, we have shown that the addition of rt-PA is mitogenic for RWP-1, BxPC-3, and PANC-1 cells and that the stable expression of the full-length cDNA in BxPC-3 and PANC-1 cells caused an increase in the proliferative abilities of these cells, which could be blocked by the inhibition of the t-PA proteolytic activity. These findings are strongly supported by the results in vivo; RWP-1 AS clones form tumors significantly smaller than controls and, reciprocally, t-PA–expressing PANC-1 P21 clones form faster-growing tumors than the same cells not expressing t-PA. The mitogenic activity of t-PA in vitro was only detected in cells growing at low serum concentration, indicating that serum is a source of active growth factors that may rescue low endogenous production of such factors.[2,37] The impaired growth of antisense t-PA clones in the subcutis in vivo, an environment with limited growth factor supply, further reinforces the notion that the endogenous production of t-PA is the relevant factor for the growth of RWP-1 cells.

t-PA itself, but not plasmin, the major product of the catalytic activity of t-PA, seems to be responsible for the stimulation of growth in the condition tested, as consistently shown by the specific inhibition produced by Pefabloc/t-PA but not by plasmin inhibitors. A number of reports by other laboratories are in agreement with our present observations.[30,34,38,39] On the other hand, our results do not allow us to conclude whether this is a direct effect, or the result of processing or induction by t-PA of other factors that would be more directly involved in the observed mitogenic effects. t-PA has been involved in the proteolytic activation of several growth factors[8,10,12]; thus, it is conceivable that its growth-stimulating effect on the cell lines used in this study is mediated by 1 or more peptide growth factors. In addition, in smooth muscle cells, platelet-derived growth factor stimulates proliferation and activates t-PA expression. In t-PA–depleted smooth muscle cells, platelet-derived growth factor is unable to stimulate cell proliferation, suggesting that t-PA can operate through other less-known mechanisms.[38]

Taken together, our observations indicate a prominent role for t-PA in the maintenance of pancreas tumor cells in a state of proliferative competence. An increasing body of evidence indicates that proteases of the extracellular matrix with well-established roles in tumor invasion, such as stromelysin 3, stromelysin 1, and matrilysin, play in addition a crucial role in early stages of tumorigenesis, promoting tumor formation and/or growth.[40–42] Evidence for a role of t-PA or u-PA and/or plasmin in the process of tumor growth varies with particular experimental models and individual tumor types. Thus, ablation of u-PA but not of t-PA causes resistance to melanoma induction,[14] the development of mammary tumors by the viral polyoma middle T antigen in plasminogen-deficient mice is not appreciably altered,[43] endogenously produced u-PA or t-PA stimulate melanoma and neuroblastoma cell proliferation in vitro,[12,44] and the combined loss of t-PA and u-PA activities results in a sig-

nificant reduction in proliferation rates of transformed endothelial cells in vitro and a reduced efficiency in tumor formation.[15] These observations lend support to our conclusion, based on the present direct evidence, that endogenous production of t-PA is necessary for the optimum growth of pancreas cancer cells in vitro and in vivo.

The second relevant new finding reported here is the effect of t-PA on the formation of the angiogenic network associated with tumor growth. Eventually, the growth of a tumor depends on the availability of nutrients in the local microenvironment, which in turn requires the development of an adequate vascular network. Formation of functional microvessels is a stepwise process in tumor progression that includes endothelial cell migration and/or proliferation.[45] In pancreatic tumors, production of angiogenic factors by neoplastic cells, including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, hepatocyte growth factor, and PAs,[21,46,47] can stimulate endothelial cell proliferation and migration. In this way, a paracrine loop of reciprocal activation could be established between tumor and endothelial cells, which permits neovascularization and, together with the perfusion of nutrients and oxygen, permits growth and progression of the tumor. Studies in vitro by others have indicated that t-PA regulates angiogenesis by stimulating endothelial cell tube formation.[34–36] Our observations provide the first in vivo evidence showing that the tumor-produced t-PA is required for the correct development of angiogenesis at the tumor site. Although the proportion of isolated endothelial cells in the tumors derived from RWP-1 control and AS clones is similar, the well-developed vascular network of the control tumors stands in marked contrast with the scant vessels of the AS tumors. These results indicate that the tumor-produced t-PA is required for the phase of endothelial tube formation and/or maturation but not for the recruitment of endothelial cells. The recent observation that t-PA might be the target protein inactivated by maspin[48,49] and by angiostatin,[50] both effective inhibitors of angiogenesis, supports our findings. The mechanisms by which t-PA would facilitate or induce the formation of tumor-associated vessels are not clear at present. t-PA may facilitate the availability of growth factors present in the extracellular matrix for endothelial cell usage,[11,51] or it may control matrix degradation to allow the formation of capillary structures.[37]

Tumors derived from RWP-1 AS clones were moderately differentiated with respect to control tumors (poorly differentiated), suggesting that a change in the endogenous t-PA levels can affect the cell-differentiation program. Tumor progression in carcinomas has been suggested to imply a gradual transition from an epithelial- to a mesenchymal-like phenotype; several growth factors, including hepatocyte growth factor, fibroblast growth factor 1, epidermal growth factor, and transforming growth factor α, which are also overexpressed in pancreatic adenocarcinomas,[47] have been implicated in this process.[52] Thus, it is conceivable that t-PA availability, allowing the activation of latent growth factors, could also affect the cell-differentiation program and tumor progression.

In conclusion, the findings reported in this study support an extension of the previously known role of t-PA in tumor invasion to an earlier phase of tumorigenesis in the pancreas, the initial stages in the growth of the primary tumor when cell proliferation and activation of angiogenesis are required. At later stages in which invasion and metastasis occur, t-PA and other proteases, such as u-PA, might be determinant. Future therapeutic strategies aimed at the inhibition of t-PA might be useful adjuvants to use in addition to conventional therapies in these tumors.

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