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The plasminogen activator system in pancreas cancer: role of t-PA in the invasive potential *in vitro*

Rosanna Paciucci, Montserrat Torà, Víctor M Díaz and Francisco X Real

Unitat de Biologia Cellular i Molecular, Institut Municipal d'Investigació Mèdica, 08003-Barcelona, Spain

Plasminogen activators (PAs) play an important role in tumor cell invasion. We have analysed the expression of tissue-type PA (t-PA), urokinase-type PA (u-PA), and their respective receptors, annexin II and u-PAR, in normal and neoplastic cultures of pancreatic cells, as well as in pancreatic tissues, and have examined their role in tumor invasiveness *in vitro*. Using Northern blotting, Western blotting, and ELISA, t-PA is detected in cultured pancreas cancer cells displaying a well differentiated phenotype but it is undetectable in less differentiated cells and in normal pancreatic cultures. In contrast, u-PA transcripts, protein, and enzymatic activity are detected both in cancer cells and in normal cultures. Higher levels of u-PAR and annexin II are present in cancer cells than in normal cultures and, in SK-PC-1 cells, both receptors are localized in the basolateral membrane. *In vitro* invasion assays indicate that both t-PA and u-PA contribute to the invasiveness of SK-PC-1 cells through reconstituted extracellular matrix. To determine the relevance of these studies to pancreas cancer, immunohistochemical assays have been used to examine the expression of t-PA, u-PA, and their receptors in normal and neoplastic tissues. t-PA is absent from normal pancreas and from tumor associated pancreatitis, whereas it is detected in the majority of pancreas cancer tissues (16/17). Annexin II is also overexpressed in some tumors (5/13). u-PAR is overexpressed in most tumor samples examined (14/15), while u-PA is weakly detected in a low number of cases (3/14); both u-PAR and u-PA are overexpressed in areas of tumor associated pancreatitis. Indirect evidences indicate that K-*ras* and p53 mutated proteins can regulate the expression of PAs. In pancreatic cancer we have found an association between codon 12 K-*ras* mutations and t-PA expression ($P = 0.04$). These results support the contention that, in the exocrine pancreas, activation of t-PA is more specifically associated to neoplastic transformation and to the invasive phenotype, whereas the induction of u-PA/u-PAR system might be more relevant to inflammatory or non-neoplastic events.

Keywords: tissue-type plasminogen activator; urokinase-type plasminogen activator; urokinase receptor; annexin II; invasion; pancreas cancer

Introduction

Cancer of the exocrine pancreas is a highly aggressive tumor: it represents the fourth cause of cancer death in the United States and its 5-year survival rate is less than 2% (reviewed in Warshaw, 1992). The causes of its clinical behavior are not known. The tumor's biological features, genetic or epigenetic, may be at the basis of its aggressiveness. Alternatively, certain characteristics of the pancreas, such as the paucity of the connective tissue or the lack of a capsule, may also participate. The majority of exocrine pancreatic tumors are classified as 'ductal adenocarcinomas' on the basis of their microscopic appearance. The study of the molecular biology of these tumors has revealed that quantitative and qualitative changes in gene expression occur during their evolution. Point mutations in K-*ras* (Smit *et al*., 1988; Hoorens *et al*., 1993; Berrozpe *et al*., 1994; Caldas *et al*., 1994b), deletions and mutations in p53 (Hollstein *et al*., 1991; Hoorens *et al*., 1993), MTS-1 (Caldas *et al*., 1994a) and DPC-4 (Hahn *et al*., 1996), and overexpression of several genes potentially involved in tumor establishment and progression have been described (reviewed in Friess *et al*., 1996). Epigenetic changes, i.e. the overexpression of growth factor receptors and their ligands such as c-*met*/HGF, can lead to the activation of autocrine and paracrine loops enhancing protease secretion, tumor cell motility, and invasiveness *in vitro* and in experimental models (Bellusci *et al*., 1994; Jeffers *et al*., 1996; Burfeind *et al*., 1996; Paciucci *et al*., submitted). The biological relevance of these alterations is suggested by their association with a worse prognosis (reviewed in Friess *et al*., 1996).

To get further insight into the molecular basis of pancreas cancer, we set to identify cDNAs overexpressed in SK-PC-1 pancreas cancer cells but not in normal pancreas tissue using a subtractive hybridization technique. Preliminary results using this strategy suggested that overexpression of tissue-type plasminogen activator (t-PA) might be relevant to this tumor (Paciucci *et al*., 1996). The plasminogen proteolytic system plays a central role in tumor cell invasion mediated by secreted proteases (Mignatti *et al*., 1986). In addition to its recognized role in degrading extracellular matrix proteins, plasmin can activate several latent metalloproteases relevant for malignant cell invasion (He *et al*., 1989; Knauper *et al*., 1996) and plasmin or PAs can activate latent growth factors like HGF and TGFβ which may contribute to tumor development and spreading (Sato and Rifkin, 1989; Lyons *et al*., 1990; Mars *et al*., 1993; Naldini *et al*., 1992). t-PA, and especially urokinase (u-PA), have been shown to be overexpressed in a variety of human tumors (reviewed in Dano *et al*., 1985, and in Kwaan, 1992). The complex formed by u-PA bound to its membrane receptor (u-PAR) is an important component of the cell migration machinery, providing an inducible, localized cell surface proteolytic activity that

Correspondence: R Paciucci
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is related to the invasive properties of cells (reviewed in Blasi, 1996). Thus, increased expression of u-PA and u-PAR in tumor cells has been correlated with increased invasive potential *in vitro* and metastatic activity *in vivo* (Ossowski, 1988; Ossowski *et al*., 1991; Pyke *et al*., 1995; Wei *et al*., 1996). Furthermore, an increased production of u-PA and u-PAR is associated with a poor prognosis in breast cancer (Nielsen *et al*., 1996) and other tumors (Hsu *et al*., 1995; Verspaget *et al*., 1995). By contrast, there is less evidence for a role of t-PA in tumor progression, though its overexpression has also been reported in selected tumor types (Wilson *et al*., 1983; Wilson and Francis, 1987; De Vries *et al*., 1994; Moser *et al*., 1994). The mechanism of action of t-PA in invasive processes is not completely clear. Its interaction with an endothelial cell membrane receptor specific for t-PA/plasminogen, identified as annexin II, is likely to be involved in endothelial cell migration (Hajjar *et al*., 1994, 1996). In tumor invasion, no data are available to confirm a similar mechanism and no reports have analysed the expression of annexin II in relationship with t-PA production.

To obtain further information about the contribution of t-PA to the aggressiveness of pancreatic tumors, we have taken advantage of the availability of a panel of well characterized pancreas cancer cell lines (Vilá *et al*., 1994a, 1995a) and of normal exocrine pancreas cultures (Vilá *et al*., 1994b). We have investigated the expression of t-PA, u-PA and their receptors in these cells and have assessed the relative contribution of the two PAs to the invasive phenotype *in vitro*. In addition, we have analysed the expression of the PA system components in normal and neoplastic pancreas tissues. Our findings support that t-PA overexpression is selectively associated with neoplastic transformation of pancreatic epithelial cells and may contribute to their invasive potential.

Results

Expression of proteases in cultured human pancreatic cells

To investigate the expression of t-PA and u-PA genes in pancreatic epithelial cells *in vitro* we used normal primary pancreatic cultures displaying a ductal phenotype (NPC) and a panel of tumor cell lines selected on the basis of their differentiation properties: SK-PC-1, MZ-PC-2, IMIM-PC-2 and Capan-1 display a differentiated phenotype resembling that of normal duct cells, RWP-1 is moderately differentiated, and IMIM-PC-1, SK-PC-3, Hs766T, Panc-1, BxPC-3 and AsPC-1 show a less differentiated phenotype (Vilá *et al*., 1994a, and unpublished results). SW979 and SW850 cells are derived from exocrine pancreas tumors, while QGP-1 cells are derived from an endocrine tumor. The phenotypic characteristics of these three lines have not been analysed in detail. Figure 1a shows that the levels of t-PA transcripts are high in 5/5 differentiated or moderately differentiated cell lines but are undetectable in 5/6 undifferentiated cells and in NPC. In contrast, u-PA transcripts are detected both in pancreas cancer cell lines, regardless of the differentiation phenotype, and in NPC.

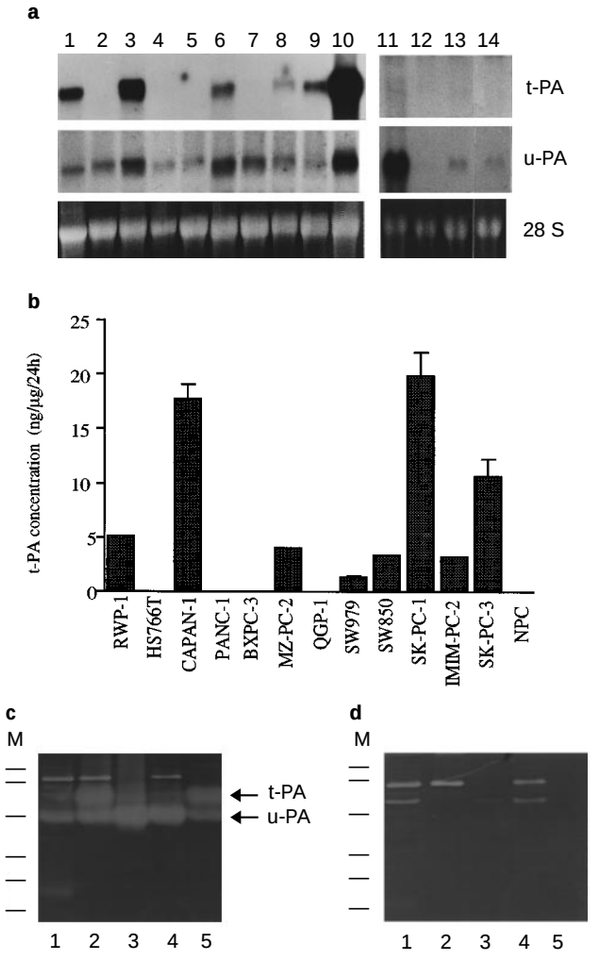

Figure 1 Expression of t-PA and u-PA transcripts in cultured pancreas cancer cells and in NPC. (**a**) Northern blotting performed with 10 μg of total RNA. Lane 1, RWP-1; 2, Hs766T; 3, Capan-1; 4, Panc-1; 5, BxPC-3; 6, MZ-PC-2; 7, QGP-1; 8, SW979; 9, SW850; 10, SK-PC-1; 11, IMIM-PC-2; 12, IMIM-PC-1; 13, SK-PC-3; 14, NPC. (**b**) ELISA assay to detect t-PA present in the conditioned medium of cultured cells. Levels of t-PA are expressed as ng of t-PA/μg of protein in cell lysate/24 h. (**c**) and (**d**) Representative zymographic analysis of secreted proteases performed with 10 μl cell culture supernatant. 1, NPC; 2, SK-PC-1; 3, IMIM-PC-2; 4, IMIM-PC-1; 5, SK-PC-3 cells. (**c**) Zymographic analysis performed in plasminogen/gelatin containing gels to determine the presence of plasminogen activators. (**d**) Zymographic analysis performed in gelatin containing gels to determine the presence of gelatinases. M, Molecular weight markers: 112, 84, 53, 35, 21, and 15 kDa

Protease secretion in the conditioned medium of cultured cells was evaluated using ELISA assays (Figure 1b and gel zymography (Figure 1c and d). The level of secreted immunoreactive t-PA correlates well with the mRNA levels by Northern blotting. No secretion of t-PA is detected in the conditioned medium from Hs766T, Panc-1, BxPC-3, or QGP-1 cancer cells nor in NPC (Figure 1b and data not shown). Figure 1c shows that secreted u-PA enzyme, corresponding to the 54 kDa lytic band in the plasminogen-gelatin zymogram, is detected in all cell lines examined, including NPC, whereas secreted t-PA is detected in SK-PC-1, IMIM-PC-2, and SK-PC-3 tumor cells, but not in NPC. The proteolytic activity of proteins with mobilities of 92 kDa and 72 kDa

likely correspond to the two forms of MMP9/Gelatinase B and/or MMP2/Gelatinase A metalloproteases, respectively, since they are also detected in the gelatin zymogram (Figure 1d). These results indicate that the expression of t-PA, u-PA and metalloproteases is independently regulated in pancreatic cells and that, among them, only t-PA is strongly associated with the neoplastic phenotype.

Expression and membrane localization of annexin II and u-PAR in pancreatic epithelial cells

Because PA receptors may focus proteolytic activity at the cell membrane, thus increasing cell invasiveness, we examined PA receptor expression in cultured pancreatic cells. The expression of annexin II and u-PAR in lysates from pancreas cancer cells and NPC was determined by Western blotting. Annexin II is detected both in NPC and in cancer cells, with higher levels in SK-PC-1 and AsPC-1 cells (Figure 2a). u-PAR is also detected in NPC, but levels are higher in IMIM-PC-2, IMIM-PC-1, and AsPC-1 cancer cells (Figure 2b): two major components are present with a relative mobility of 45–65 kDa, likely identifying different glycosylated forms (Behrendt *et al*., 1990; Ploug *et al*., 1991).

The membrane localization of u-PAR is a controversial issue (Bohuslav *et al*., 1995; Limongi *et al*., 1995; Stahl and Mueller, 1995; Stefansson and Lawrence, 1996). To determine the localization of u-PAR and annexin II, we used SK-PC-1 cells labeled apically or basolaterally by domain selective membrane biotinylation. Cells were lysed and biotinylated surface proteins, captured with streptavidin-agarose, were identified by Western blotting (Figure 2c). Both PA receptors are exclusively detected in the basolateral membrane compartment. MUC1 and epidermal growth factor receptor (EGFr), used as controls for apical (Carrato *et al*., 1994) and basolateral proteins, respectively, showed the expected membrane distribution.

Therefore, PA receptors are expressed at higher levels in tumor pancreas cells, and are specifically localized at the basolateral membrane.

SK-PC-1 cell invasiveness in vitro *depends on both t-PA and u-PA activity*

We determined the invasive potential of SK-PC-1 cells expressing high levels of t-PA and u-PA using Matrigel-coated Transwell filters. The blockade of the proteolytic activity of each of these proteases by using specific inhibitors allowed us to determine their individual contribution to the process of cell invasion. Figure 3 shows that, in the presence of neutralizing antibodies to t-PA, cell invasiveness is reduced to $55.2 \pm 21\%$ ($P = 0.018$); this effect is not observed upon incubation with control antibodies ($95.3 \pm 13.4\%$, $P = 0.83$). Pefabloc/t-PA (15 μM), a synthetic derivative of benzamidine that selectively inhibits t-PA activity (Stürzebecher and Markwardt, 1988) reduces invasion to $54.3 \pm 14.5\%$ ($P = 0.05$). The simultaneous addition of anti-t-PA antibody and Pefabloc/t-PA further decreases cell invasiveness to $32 \pm 21\%$ of control ($P = 0.05$). In the presence of amiloride (0.2 mM), a selective inhibitor of u-PA activity (Vassalli and Belin, 1987), invasiveness is reduced to $40.2 \pm 19.9\%$ of control ($P = 0.006$). EACA decreases cell invasion to $75.2 \pm 35\%$ ($P = 0.07$), suggesting that plasminogen binding to membrane lysine residues is also necessary for this process. The combination of amiloride (0.02 mM) and Pefabloc/t-PA (15 μM) induced a significantly more potent inhibition of cell invasiveness than either of the two agents alone ($P < 0.05$); at the doses tested, an additive effect was demonstrated (data not shown). Thus, both t-PA and u-PA proteolytic activities contribute to the invasive capacity of SK-PC-1 cells *in vitro*.

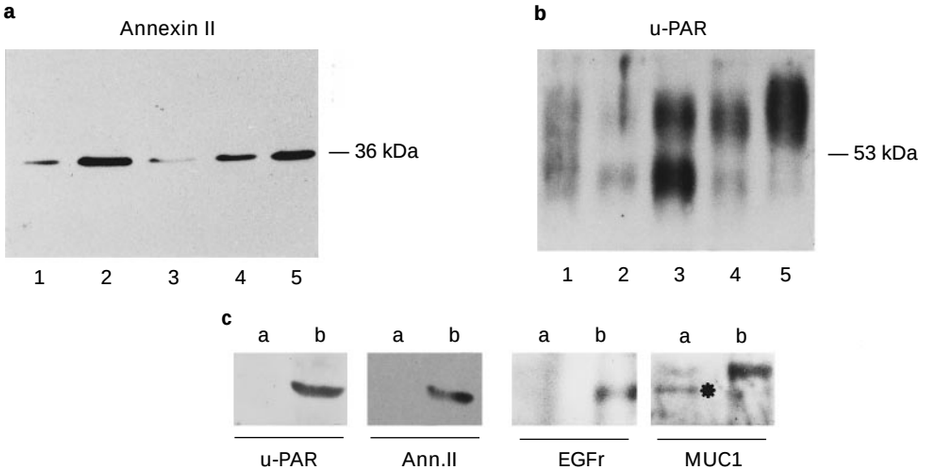

Figure 2 Expression and membrane localization of annexin II and u-PAR in pancreatic epithelial cells. (**a**) Western blotting analysis of annexin II using total cell lysates (50 μg). (**b**) Western blotting analysis of u-PAR using glycoproteins isolated from 200 μg of total cell lysate. Lane 1, NPC; 2, SK-PC-1; 3, IMIM-PC-2; 4, IMIM-PC-1; 5, AsPC-1. (**c**) SK-PC-1 polarized cells were cultured on Transwells and domain-selective biotinylated as described in Materials and methods. Cell lysates were fractionated with streptavidin-agarose and biotinylated proteins were revealed by Western blotting with specific antibodies detecting u-PAR (u-PAR), annexin II (Ann. II). Replica filters were used to detect apical (MUC1) (*) and basolateral (EGF receptor) control proteins. (a) apical, and (b) basolateral membrane protein preparations. The high molecular weight band detected with MUC1 antibody corresponds to a non-specific component migrating at the separation between the stacking and the resolving gels

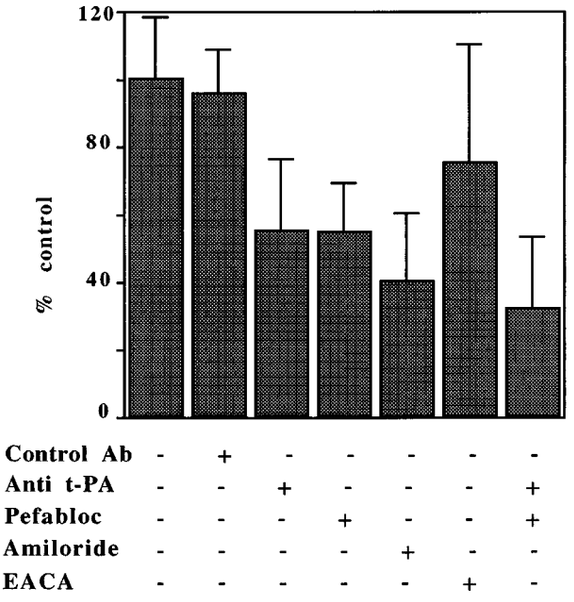

Figure 3 *In vitro* cell invasion assay. SK-PC-1 cancer cells were cultured on Matrigel-coated Transwell filters for 72 h in the absence (control) or in the presence of the indicated factors. Quantitative determinations of cells invading the bottom chamber were performed with ^{3}H-labeled cells as described in Materials and methods. All experiments were performed at least twice and in triplicate samples. For statistical analysis, see the text

Immunohistochemical detection of PAs and their receptors in pancreatic tumors

To confirm the results obtained with cultured cells, we investigated the expression of PAs and their receptors in frozen normal and tumor tissues by the immunoperoxidase technique. A summary of the findings is presented in Table 1 and representative results are shown in Figures 4 and 5.

In normal pancreas tissue, all epithelial cells in the exocrine and endocrine components are unreactive with anti-t-PA antibodies (Figure 4). In these assays, a strong reaction with vascular endothelial cells is observed, as previously reported (De Vries *et al*., 1994). In contrast, immunoreactive t-PA is detected in 16/17 ductal-type adenocarcinomas and expression is localized both in the cytoplasm and in the lumen of tumor glands (Figure 4b). In normal tissue, annexin II is weakly detected in connective tissue fibroblasts surrounding normal ducts but not in epithelial cells (Figure 4c). However, in tumors (Figure 4d), annexin II is detected in neoplastic cells in 5/13 cases (Table 1).

Anti u-PA antibodies are unreactive with all cell types in the normal exocrine and endocrine pancreas (Figure 5a) and are weakly reactive with 3/14 ductal adenocarcinomas (Figure 5b). By contrast, u-PAR is weakly detected in normal acinar and ductal cells (Figure 5d) but it is overexpressed in the majority of tumors examined (14/15) (Figure 5e).

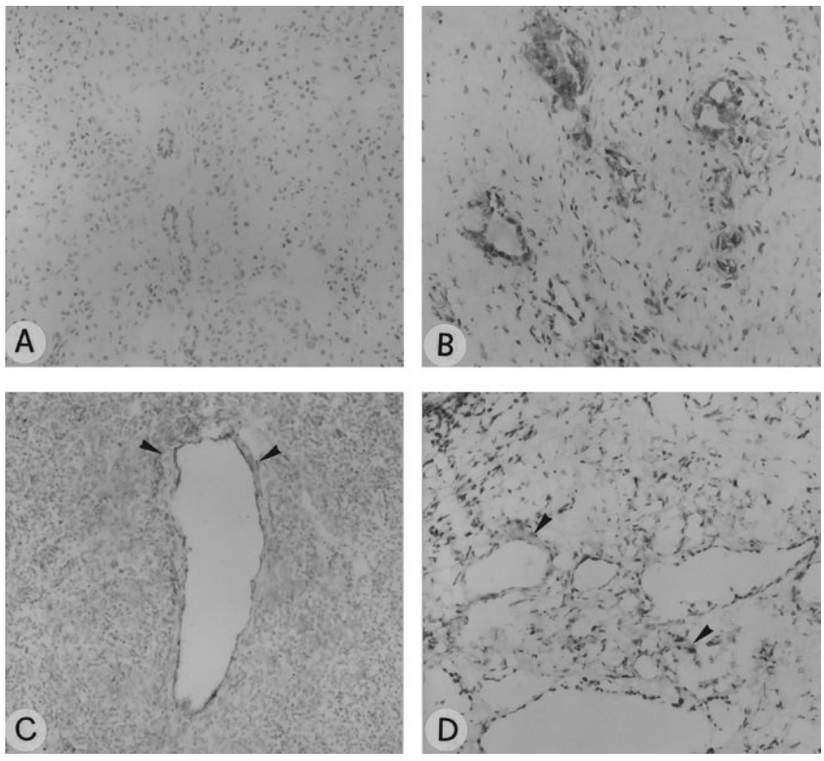

Figure 4 Expression of t-PA and annexin II in normal and pancreas tumor tissues analysed using indirect immunoperoxidase. (**a**, **c**) normal pancreas; (**b**, **d**) pancreas cancer. (**a**, **b**) t-PA: no reaction is present in normal acinar or duct cells, nor in stromal cells, while tumor cells are strongly reactive. (**c**, **d**) annexin II: a weak positive reaction is detected in mesenchymal cells around ducts in normal pancreas, while tumor cells (arrowheads in **d**) show heterogeneous reactivity. No reactions were detected with control antibodies. Original magnification: ×200 (**a**, **b**, **d**), ×100 (**c**)

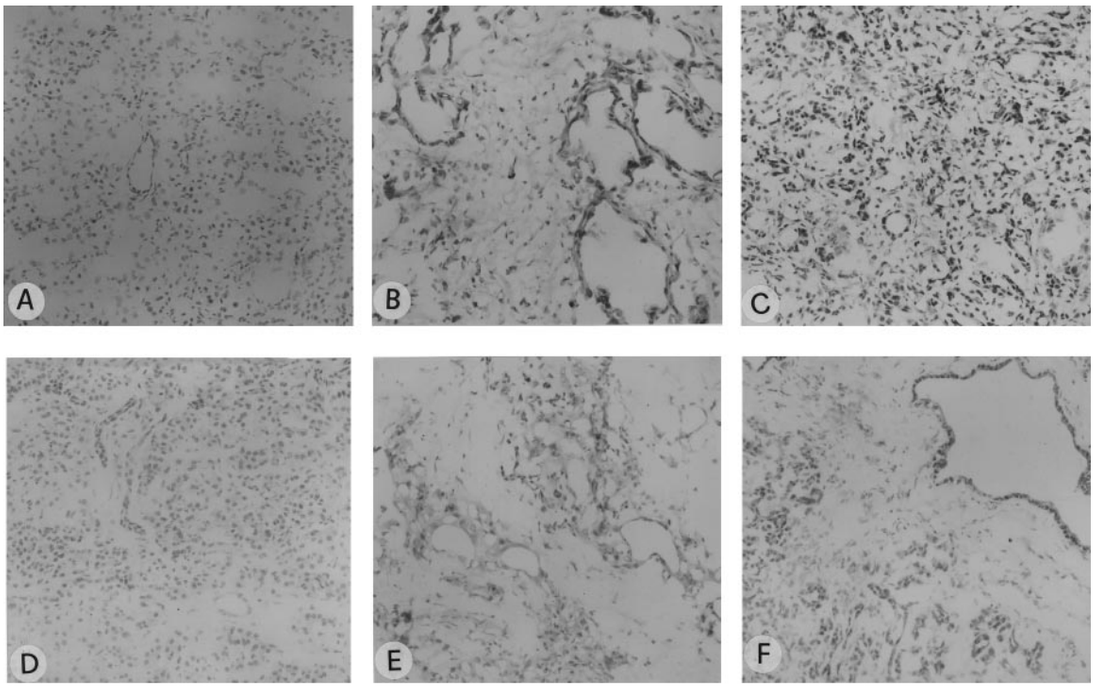

Figure 5 Expression of u-PA and u-PAR in normal pancreas, pancreas cancers, and tumor-associated obstructive pancreatitis. (**a**, **d**) normal pancreas, (**b**, **e**) pancreas cancer, (**c**, **f**) obstructive pancreatitis adjacent to pancreas cancer. (**a**–**c**) u-PA: no reaction is detected in normal pancreas; u-PA is detected only in some pancreatic tumors but it is strongly expressed in areas of obstructive pancreatitis. (**d**–**f**) u-PAR: a weak positive reaction is detected in normal acinar and ductal cells and a strong reaction is detected in tumor cells and in areas of pancreatitis. No reactions were detected with control antibodies. Original magnification ×200

Because most samples examined corresponded to primary tumors and were moderately differentiated, it was not possible to determine an association of the expression of PAs and their receptors with tumor progression or differentiation degree (data not shown).

To determine if the overexpression of t-PA and u-PAR is restricted to tumor cells, areas of chronic obstructive pancreatitis adjacent to pancreatic cancers were also examined. As shown in Table 1, t-PA and annexin II are generally undetectable in pancreatitis, whereas u-PA and its receptor are strongly expressed in these areas (Figure 5c and f). These results indicate that pancreatic tumors have increased expression of t-PA and u-PAR; however, while the former is restricted to pancreas cancer cells, the latter is not.

Table 1 Expression of components of the t-PA/u-PA proteolytic system in exocrine pancreas

Ab	*Normal pancreas*[a,b]	*Pancreas cancer*[a]			*Pancreatitis*
		+[c]	++/+++[c]	%	
t-PA	0/6	3/17	13/17	94	1/5
u-PA	0/7	3/14	0/14	21	4/4
Annexin II	0/7	2/13	3/13	38	1/5
u-PAR	8/9	6/15	8/15	93	8/8

[a] Reactive samples/total number of samples tested. [b] Reactivity in acini and ducts. [c] Reactions scored as described in Materials and methods

Association of t-PA expression and genetic alterations in pancreas cancer

Because there is indirect evidence indicating that PA promoters can be regulated by signal transduction pathways involving ras and p53 (Axelrod *et al*., 1989; Jankun *et al*., 1991; Lengyel *et al*., 1995), we investigated the association of t-PA expression and K-*ras* or p53 mutations. K-*ras* and p53 mutations in pancreatic tumors and cell lines were previously determined (Kalthoff *et al*., 1993; Berrozpe *et al*., 1994, and unpublished results). Table 2 shows that 6/8 cell lines secreting high levels of t-PA contain K-*ras* codon 12 mutations while the latter are present only in 1/4 lines that lack t-PA secretion. In addition, 9/12 tumors expressing t-PA contain K-*ras* codon 12 mutations. A combined analysis of the association between t-PA production and K-*ras* mutations in cell lines and tumors showed a significant association ($P = 0.04$). Regarding p53 mutations, no association with t-PA production was observed ($P = 0.586$). These findings suggest that K-*ras* gene mutations may contribute to the activation of t-PA expression in exocrine pancreatic cancer.

Discussion

The major novel findings described in this paper can be summarized as follows: first, t-PA is commonly expressed in pancreas cancer tissues and cell lines and appears to be selectively associated with the neoplastic phenotype; second, *in vitro*, both t-PA and u-PA contribute to the invasive behavior of pancreas cancer cells; third, in the neoplastic pancreas, concomitant expression of u-PA and its receptor are mainly associated with areas of pancreatitis; fourth, K-*ras*

Table 2 Mutations in p53 and K-*ras* genes and t-PA expression in pancreatic tumors

Sample	*p53*[a] *mutation*	*K*-ras[a] *mutation (codon 12)*	*t-PA expression*[b]
Cell line			
SK-PC-1	+	+	20
CAPAN-1	+	+	17.8
SK-PC-3	+	+	10.5
RWP-1	–	+	5.1
MZ-PC-2	+	+	4.0
IMIM-PC-2	+	+	3.3
SW850	–	–	3.2
SW979	–	–	1.4
Hs766T	+	–	b.d.
Panc-1	+	+	b.d.
BxPC3	+	–	b.d.
QGP-1	–	–	b.d.
Tumor			
44139	–	+	+
M-726	n.d.	+	+
M-651	n.d.	+	+
907072	+	+	+
9	–	+	+
M-82	–	+	+
1	–	+	+
42812	–	+	+
M-186	+	+	+
M-148	+	–	+
M-692	n.d.	–	+
IEMOLA	–	–	+
M-621	n.d.	–	–

[a] From Berrozpe *et al.* (1994), Kalthoff *et al.* (1993), and unpublished results. [b] Expression in pancreatic cancer cell lines and tumors determined by ELISA (nanograms of secreted t-PA/24h/micrograms of total cellular protein) and immunohistochemistry respectively. b.d. Below detectable levels. n.d. Not done

gene mutations may participate in the activation of the expression of t-PA in these tumors.

There is little information regarding the expression of the plasmin proteolytic system components in exocrine pancreatic cancer. The results from our studies indicate that expression of PAs in these tumors differs considerably from the findings reported in other tumor types. Thus, t-PA but not u-PA is the most commonly expressed PA in pancreas tumors and it is detected in tumor cells rather than in the stroma. In contrast, in breast and colon cancer, for example, u-PA is mainly produced by stromal cells whereas u-PAR is mostly produced by cancer cells and macrophages (Nielsen *et al*., 1996; Pyke *et al*., 1991). Although *in vitro* both proteases contribute to cellular invasion, our findings suggest that t-PA is the main PA associated with the neoplastic process while u-PA/u-PAR, which are detected in tumor as well as in non-neoplastic epithelial cells, would be more likely involucrated in tissue remodeling.

Regarding its mechanism of action, t-PA has been mainly implicated in fibrinolysis because it is dependent on fibrin binding for its action (Carmeliet *et al*., 1994). Nevertheless, deposition of fibrin in tumors is a common event (Dvorak, 1986) and, given the important desmoplastic reaction characteristic of pancreas cancer, overexpression of t-PA might be an effective proteolytic pathway of tumor invasion in this organ. t-PA can also act through cell surface receptors: annexin II in endothelial cells (Hajjar *et al*., 1994) and amphoterin, a member of the high mobility group 1 proteins, in neuroectodermal cells (Parkkinen and Rauvala, 1991). Annexin II is a peripheral membrane protein bound to phospholipids through calcium dependent interactions and it functions as a receptor for both t-PA and plasminogen, thus focalizing and amplifying the activity of this protease on the surface of endothelial cells (Cesarman *et al*., 1994; Hajjar *et al*., 1994, 1996). Increased expression of annexin II has been reported in several tumor types, including pancreas tumor cell lines (Vishwanatha *et al*., 1993; Davis and Vishwanatha, 1995). It is not clear where annexin II is exactly located in the cell (reviewed in Moss, 1997). The demonstration that t-PA is implicated in *in vitro* matrix invasion of pancreas cancer cells and that invasive cells express high levels of annexin II localized at the basolateral membrane, favour a yet undescribed mechanism of tumor invasion mediated by t-PA bound to its surface receptor. The co-expression of t-PA and amphoterin at the leading edge of growing neurites (Parkkinen and Rauvala, 1991; Parkkinen *et al*., 1993) and the t-PA-stimulated migration of PC12 cells (Pittman and Di Benedetto, 1995) suggest a similar mechanism for extracellular matrix invasion.

In agreement with previous reports describing u-PAR and u-PA at focal contact points (Pollanen *et al*., 1987, 1988) or u-PAR association with β1 and β2 integrins (Bohuslav *et al*., 1995; Stefansson and Lawrence, 1996; Wei *et al*., 1996) we found u-PAR to be localized in the basolateral membrane in SK-PC-1 cells. This finding does not exclude the presence of the receptor in other membrane compartments, such as caveolae (Stahl and Mueller, 1995; Limongi *et al*., 1995; Paciucci *et al*., submitted), where, possibly, protein availability for surface biotinylation reactions is decreased. In this respect, we have shown that in IMIM-PC-2 cells the proteolytically activated u-PAR (Resnati *et al*., 1996) is not localized in caveolae and corresponds to the form associated with the motile phenotype (Paciucci *et al*., submitted). Thus, we suggest that u-PAR localized in the basolateral membrane is the form involved in *in vitro* invasion of pancreas tumor cells.

With respect to the mechanisms responsible for u-PA and u-PAR overexpression in peritumoral pancreatitis, it is possible that the inflammatory cells present in this lesion contribute to their activation. The u-PA and u-PAR promoters can in fact be activated by phorbol esters, cytokines, such as TNFα (reviewed by Medcalf *et al*., 1990; Kwaan, 1992; Wang *et al*., 1994), TGF β1, EGF (Lund *et al*., 1991; Medcalf *et al*., 1991) and HGF (Pepper *et al*., 1992; Jeffers *et al*., 1996). Further studies are needed to establish whether u-PA/u-PAR overexpression in pre-neoplastic pancreatic lesions play a role in early stages of tumor progression.

One question raised by our study is the molecular basis of t-PA activation of expression in pancreas cancers. Cells transformed by activated ras proteins overexpress u-PA and t-PA and show enhanced invasion and metastatic abilities, while cells containing mutated p53 proteins lose the ability to repress the expression of both t-PA and u-PA, suggesting that the genetic alterations present in tumors may participate in activating PA expression (Axelrod *et al*., 1989; Jankun *et al*., 1991; Kunz *et al*., 1995; Lengyel *et al*., 1995). These evidences, together with the association between codon 12 K-*ras* mutations and t-PA expression

described here, suggest that K-*ras* activation is one of the mechanisms contributing to t-PA induction in pancreatic cancer.

The role t-PA in tumor cell invasion needs to be explored *in vivo*. Our findings suggest that the contribution of t-PA overexpression to the aggressive behavior of pancreatic tumors may be severalfolds: (i) through direct activation of growth factors; (ii) through activation of plasmin, which, in turn, activates growth factors and latent metalloproteases shown to be produced in these tumors (this work and Gress *et al*., 1995); (iii) through activation of plasmin which directly degrades extracellular matrix proteins and allows tumor cell invasion. Considering its anatomical characteristics, these mechanisms may be sufficient to allow invasion in the pancreas while additional ones might be necessary for invasion in other tissues, such as colon, where the tumor has to invade a thick muscle layer. The results reported here emphasize the need to examine the role of t-PA in pancreas cancer progression in greater depth.

Materials and methods

Cell culture and reagents

IMIM-PC-1 and IMIM-PC-2 cell lines were established at the Institut Municipal d'Investigació Mèdica (Barcelona, Spain) and SK-PC-1 and SK-PC-3 were established at the Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center (New York, USA) (Vilá *et al*., 1995a). SW850, SW979 and QGP-1 cells were obtained from Dr H Kalthoff (Christian-Albrechts-University, Kiel, Germany). Other cell lines were obtained from the American Type Culture Collection (Rockville, MD). All cell lines are derived from ductal adenocarcinomas, except for QGP-1 which is from endocrine origin. Pancreas cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL) at 37°C in 5% CO_2 atmosphere. Fresh normal pancreas tissue was obtained from organ donors and processed as described in order to isolate the exocrine component, and the characteristics of normal exocrine pancreas cultures (NPC) have been described (Vilá *et al*., 1994b). Matrigel was purchased from Becton Dickinson (Bedford, MA), amiloride was from Sigma (St Louis, MO), and Pefabloc/t-PA was purchased from Pentapharm (Basel, Switzerland).

Antibodies

Neutralizing goat antibodies recognizing u-PA (ref. 398) or t-PA (ref. 387) were purchased from American Diagnostica (Greenwich, CT). Rabbit anti-u-PAR antibody was a kind gift of Dr D Talarico (Ospedale San Raffaele, Milano, Italy). Mouse monoclonal anti-annexin II antibody was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal serum detecting EGFr (4/17) was obtained from Dr S Decker (Rockefeller University, NY), and mouse anti-MUC1 monoclonal antibody BC3 was provided by Dr IFC McKenzie (Austin Research Institute, Heidelberg, Australia). Peroxidase-coupled goat antibodies detecting mouse Ig or rabbit Ig were purchased from Dakopatts (Glostrup, Denmark). Biotin-labeled mouse anti-goat Ig was from Pierce (Rockford, IL) and peroxidase-coupled streptavidin was from Zymed (San Francisco, CA). Control normal goat serum was from Sigma.

Northern blot analysis

Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Sacci and Chomczynski, 1987). Total RNA (10 μg) from cultured cells or tissue was fractionated in 1% agarose-formaldehyde gels, transferred to nylon filters, and hybridized to probes labeled with α^{32}PdCTP by random priming (Feinburg and Volgelstein, 1983). Hybridizations were carried out overnight at 42°C, followed by washing with $0.2 \times$ SSC and 1% SDS at 60°C followed by autoradiography. The quality and quantity of the transferred RNA were assessed by ethidium bromide staining of ribosomal RNAs.

Enzyme-linked immunosorbent assay (ELISA)

Maxisorb plates (Nunc, Naperville, IL) were coated with conditioned serum-free medium, washed with PBS, and incubated with gelatin (1% in PBS). Anti-t-PA antibodies (2 μg/ml in 0.1% Tween 20 in PBS) were added for 1 h at 37°C and plates were washed with PBS/Tween. Biotin-labeled mouse anti-goat IgG (diluted 1/50 000 in PBS/Tween) was added for 1 h at 37°C and, after washing, alkaline phosphatase-coupled streptavidin (Zymed) was added for an additional 1 h at 37°C (1/4000 dilution in PBS/Tween). After washing, reactions were developed using 4-methylumbelliferyl phosphate (Boehringer Mannheim, Barcelona, Spain) (1 mg/ml in triethanolamine buffer pH 9.5). Purified t-PA (Actilyse, Boehringer Mannheim) was used as a standard. A Cytofluor 2300 system (Millipore, Bedford, MA) was used to measure the reaction products.

Western blotting

Cells from preconfluent cultures were lysed at 4°C in 20 mM Tris pH 6.8, 1% Triton X-100, 3 mM $MgCl_2$, 50 mM NaCl and 300 mM sucrose. After centrifugation for 10 min at 13 000 *g*, proteins from the soluble fraction (50 μg) were fractionated by SDS–PAGE and transferred to nitrocellulose filters. To enrich for glycosylated proteins, cell lysates (200 μg of protein) were fractionated by affinity chromatography on Concanavalin A-Sepharose equilibrated in buffer A (10 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP40). Glycoproteins were eluted with buffer A supplemented with 1 M α-methyl-mannoside. The rest of the procedure was carried out as previously described using a peroxidase-labeled secondary antibody (Vilá *et al*., 1995b).

Domain-selective biotinylation assays

Selective biotinylation of SK-PC-1 cells was performed as described (Lisanti *et al*., 1990). Briefly, cells were seeded in 0.4 μm pore Transwells (Costar) until confluence. When the monolayer was impermeable, cells were washed with cold Dulbecco's PBS (D-PBS), and sulfo-NHS-biotin (Pierce) (0.5 mg/ml) was added to the apical or basolateral compartment for 20 min at 4°C. After washing with cold D-PBS, cells were incubated with DMEM without FBS for 15 min at 4°C, washed with cold D-PBS, lysed in 1% Triton X-100, 50 mM Tris-HCl pH 8, 62.5 mM EDTA supplemented with protease inhibitors, and fractionated with streptavidin-agarose. Biotinylated proteins were resolved by 9% SDS–PAGE and identified by Western blotting using specific antibodies. Antibodies recognizing MUC1 and EGFr were used as controls of the apical and basolateral selective biotinylation reactions.

Zymography

Twenty-four hours conditoned medium from cells cultured in DMEM without FBS was centrifuged at 13 000 *g* for

15 min at 4°C. Sample volumes to be used in the zymography assay were determined on the basis of protein concentration in the soluble fraction of the cell lysates and proteins were separated by 10% SDS–PAGE in plasminogen- and/or gelatin-containing gels, as described elsewhere (Heussen and Dowdle, 1980). The protease activity was revealed by incubating the gels in 2.5% Triton X-100 for 1 h followed by incubation in 0.1 M glycine pH 8.3 overnight at 37°C. After fixing proteins with methanol/acetic acid/water (30 : 10 : 60), gels were stained with 0.1% amido black and destained.

Immunohistochemical methods

Normal pancreas tissue was obtained from organ donors and tumor samples were obtained from surgical specimens. Tissues were snap frozen in liquid nitrogen or in isopenthane cooled at −80°C and stored at −80°C until used. Five micron sections of frozen tissues were fixed in cold acetone for 10 min and used in immunohistochemical assays. The indirect immunoperoxidase method was used, as described elsewhere (Carrato *et al*., 1994). Reactions were scored as (+++) when staining was clearly seen at the ×100 magnification, (++) when it was clearly seen ×200 magnification, and (+) when definite staining could only be seen at the ×400 magnification. In all assays, control normal goat serum or an irrelevant mouse mAb were used and found to be unreactive. The concentration of antibodies used in these assays were: anti-t-PA, 10 μg/ml; anti-u-PA, 5 μg/ml; anti-annexin II, 15 μg/ml; anti-u-PAR, 1 : 500 dilution.

In vitro *invasion assays*

The invasive potential of cultured tumor cells was tested using Matrigel-coated Transwell filters (Costar, Cambridge, MA). Matrigel diluted 1 : 20 in PBS, was used to coat filters (50 μl) and allowed to dry overnight under u.v. light. Cells (4×10^4) were plated on top of Matrigel in DMEM witout FBS supplemented with 1% BSA. DMEM containing 10% FBS was placed on the bottom wells. Specific protease inhibitors were added to both top and bottom wells. To inhibit t-PA proteolytic activity neutralizing anti-t-PA antibody (150 μg/ml) or Pefabloc/t-PA (15 μM) were added; u-PA proteolytic activity was inhibited by adding 0.2 mM amiloride, and plasminogen binding to cell membranes was competed by addition of EACA (50 mM). Anti t-PA antibody was tested at 100 and 150 μg/ml and no toxicity on cells was detected at these concentrations. Amiloride at the concentration of 0.02, 0.2, and 2 mM, EACA at 10, 50 and 150 mM and Pefabloc at 15, 50 and 100 μM were determined not to be toxic for the cells. Plates were incubated at 37°C for 72 h and cells that invaded Matrigel and passed to the lower compartment of the filters were visualized by fixation with 1% glutaraldehyde and staining with 0.2% crystal violet. Quantitative determinations were obtained using overnight ^{3}H-thymidine-labeled cells (2 μCi/10^5 cells/ml) plated on top of Matrigel. After carefully removing the Matrigel and the cells present on the top part of the filters, these were cut and radioactivity was quantitated in a beta scintillation counter. All assays were carried out in triplicate and at least two independent experiments were performed.

K-ras *and p53 mutation detection*

The association between t-PA expression and K-*ras* mutations was established using data from prior studies (Kalthoff *et al*., 1993; Berrozpe *et al*., 1994) and our own unpublished data using PCR amplification and the artificial restriction fragment length polymorphism strategy (Malats *et al*., 1997). p53 mutation data, pooled from prior studies, were obtained using direct sequencing of PCR amplification products (Kalthoff *et al*., 1993; Berrozpe *et al*., 1994).

Statistical analysis

The effect of various agents on cell invasiveness, as a continuous variable, was compared with the control experimental conditions using the Mann–Whitney test. The association of t-PA expression (+ or −) and K-*ras* or p53 mutations (dichotomous variables) was assessed by Fisher's exact test.

Abbreviations

EACA, epsilon aminocaproic acid; EGF, epidermal growth factor; FBS, fetal bovine serum; HGF, hepatocyte growth factor; NPC, normal exocrine pancreatic cultures; TGF, transforming growth factor; TNF, tumor necrosis factor; t-PA, tissue-plasminogen activator; u-PA, urokinase-type plasminogen activator; u-PAR, urokinase-type plasminogen activator receptor.

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