Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro

V M Díaz, M Hurtado, T M Thomson, J Reventós, R Paciucci

Background: Overexpression of tissue plasminogen activator (t-PA) in pancreatic cancer cells promotes invasion and proliferation in vitro and tumour growth and angiogenesis in vivo.

Aims: To understand the mechanisms by which t-PA favours cancer progression, we analysed the surface membrane proteins responsible for binding specifically t-PA and studied the contribution of this interaction to the t-PA promoted invasion of pancreatic cancer cells.

Methods: The ability of t-PA to activate plasmin and a fluorogenic plasmin substrate was used to analyse the nature of the binding of active t-PA to cell surfaces. Specific binding was determined in two pancreatic cancer cell lines (SK-PC-1 and PANC-1), and complex formation analysed by co-immunoprecipitation experiments and co-immunolocalisation in tumours. The functional role of the interaction was studied in Matrigel invasion essays.

Results: t-PA bound to PANC-1 and SK-PC-1 cells in a specific and saturable manner while maintaining its activity. This binding was competitively inhibited by specific peptides interfering with the interaction of t-PA with annexin II. The t-PA/annexin II interaction on pancreatic cancer cells was also supported by co-immunoprecipitation assays using anti-t-PA antibodies and, reciprocally, with antiannexin II antibodies. In addition, confocal microscopy showed t-PA and annexin II colocalisation in tumour tissues. Finally, disruption of the t-PA/annexin II interaction by a specific hexapeptide significantly decreased the invasive capacity of SK-PC-1 cells in vitro.

Conclusion: t-PA specifically binds to annexin II on the extracellular membrane of pancreatic cancer cells where it activates local plasmin production and tumour cell invasion. These findings may be clinically relevant for future therapeutic strategies based on specific drugs that counteract the activity of t-PA or its receptor annexin II, or their interaction at the surface level.
inactivated fetal bovine serum (FBS; Gibco-BRL) at 37°C and 5% CO₂. Goat antibodies recognising t-PA were from American Diagnostica (Greenwich, Connecticut, USA); antibodies to annexin I and annexin II were from Transduction Laboratories (Lexington, Kentucky, USA); anti-ERK 1,2, from Upstate Biotechnology (Lake Placid, New York, USA); antimonous FITC biotin labelled rabbit anti-goat IgG and mouse IgGs from Dako (Glostrup, Denmark); anti-goat TRITC from Pierce (Rockford, Illinois, USA); diisopropyl fluorophosphate (DFP), goat IgGs, streptavidin-agarose, and protein G agarose beads from Sigma (St Louis, Missouri, USA); and plasminogen and recombinant t-PA (rt-PA, Actilyse) from Boehringer Mannheim (Barcelona, Spain). Peptides LCKLSD and LGKLSL were synthesised at the Serveis Científico Tècnics, Universitat Pompeu Fabra (Barcelona, Spain).

Functional cell membrane binding assay for t-PA
Interaction of t-PA with the surface of pancreatic tumour cells was studied by analysing the plasminogen activating capacity of bound recombinant t-PA (rt-PA). Briefly, cells were seeded (7 x 10⁴ cells/well) in 24 well plates and grew to confluence. Endogenous plasminogen activators were removed from cell surface with acid glycine buffer (0.05 M glycine (pH 3), 0.1 M NaCl) for three minutes and neutralised with 0.5 M HEPES (pH 7.5) and 0.1 M NaCl. Cells were incubated with different concentrations of rt-PA for 20 minutes at 37°C. Controls contained phosphate buffered saline (PBS), containing 2% bovine serum albumin (BSA, binding buffer).

To determine the linearity of plasminogen activation, fluorescence levels (relative fluorescence units (RFU)) were measured at different times after incubation with substrates at excitation and emission wavelengths of 380 nm and 480 nm, respectively, using a Fluoroskan Reader (Labsystems, Helsinki, Finland).

Equilibrium, saturation, and competition binding assays
To determine the time needed to reach equilibrium, PANC-1 cells were incubated with rt-PA (15 nM) at 37°C for different time periods, cells were washed, and plasminogen activation was determined. For equilibrium saturation binding assays, PANC-1 cells were incubated with rt-PA at different concentrations (0–500 nM) for 20 minutes, and plasmin generation was determined. To convert RFU data to t-PA protein concentration, a standard t-PA in a fibrin coupled enzymatic assay was used (Desafib-X, Biopool, Sweden).

Analyses of binding data
Equilibrium and saturation binding data were analysed by non-linear regression algorithms using Sigma Plot software (SPSS, Chicago, Illinois, USA). For saturation binding analyses, data were fit to a one or two binding site model.

Western blotting and immunoprecipitation
Western blotting was performed as described previously. For immunoprecipitation, subconfluent SK-PC-1 cells were lysed in buffer I (20 mM Tris HCL, pH 6.8, 3 mM MgCl₂, 50 mM NaCl, 300 mM sucrose, 1% Triton X-100 (v/v)) containing 1 mM phenylmethylsulphonyl fluoride and 2 μg/ml each of aprotinin, leupeptin, and pepstatin. After centrifugation (13 000 g) the pellet containing annexin II was dissolved in buffer II (50 mM Tris HCL, 150 mM NaCl, 1% NP-40, 10 mM EGTA). Proteins (400 μg) from the supernatant and pelleted fractions were used for immunoprecipitation with antiannexin II, anti-t-PA, antiannexin-I antibodies, or goat IgGs, and recovered by protein G sepharose beads or anti-goat biotinylated IgG and streptavidin agarose beads. After washing with buffer II without EGTA, beads were resuspended in Laemmli buffer, and immunoprecipitated proteins were identified by western blotting.
In vitro invasion assays

The invasive potential of cultured tumour cells was tested using Matrigel coated Transwell filters (Costar, Cambridge, Massachusetts, USA), as previously described. Briefly, 1×10⁵ cells were resuspended in DMEM supplemented with 0.1% BSA and plated on the Matrigel coated filters in the upper chamber, and medium containing 10% FBS was placed in the bottom chamber. Plates were incubated at 37 °C for 24, 48, or 72 hours, after which cells that reached the bottom surface of the filters were visualised by staining with crystal violet. Peptides LCKLSL and LGKLSL were tested on cell invasion by mixing them with cells before seeding. Quantitative analyses were performed by counting stained cells on the filters in 10 independent fields/Transwell. Invasion abilities of cells were assigned as follows: highly invasive (+++), >100 cells/field at 24 hours; moderately invasive (++), 50–100 cells/field at 48 hours; and poorly invasive (+), 50–100 cells/field at 72 hours. Quantitative analyses of stained filters were also obtained by elution in 10% acetic acid and measuring absorbance at 595 nm.

Statistics

Results are expressed as mean (SEM) and the Student's t test was used for statistical analysis. A p value of <0.05 was considered statistically significant in all cases.

RESULTS

t-PA binds specifically and saturably to the surface of pancreatic cancer cells

Because pancreatic cancer cells express plasminogen activator inhibitor 1 (PAI-1) (Díaz and colleagues and data not shown), the physiological inhibitor of t-PA that binds with high affinity and inactivates t-PA, and the t-PA/PAI-1 complex can bind to cell surface receptors, t-PA binding

Figure 2  Binding of tissue-type plasminogen activator (t-PA) to PANC-1 and SK-PC-1 cancer cells. PANC-1 (A) and SK-PC-1 (B) cells were grown until confluence and incubated with increasing concentrations of recombinant t-PA for 20 minutes. Bound t-PA was determined 15 minutes after addition of substrate. One representative experiment of four performed in triplicate is indicated. Shown is the best fit of binding models to the data. The binding constants, as revealed by non-linear regression analyses of the data, are indicated in the text.

Figure 3  Involvement of annexin II in the binding of tissue-type plasminogen activator (t-PA) to pancreatic cancer cells. Experiments were performed with confluent PANC-1 monolayers. (A) Specificity of t-PA binding was tested using as a competitor of recombinant t-PA (r-t-PA, 10 nM) t-PA inactivated with diisopropyl fluorophosphate (DFP) at increasing concentrations. (B) Specific contribution of annexin II to binding of t-PA to the surface of PANC-1 cells was studied by disrupting the interaction with a blocking peptide (LCKLSL, filled symbols) or a control peptide (LGKLSL, open symbols). Data are mean (SEM) (n = 3). Inhibition of t-PA binding by LCKLSL was statistically significant (p = 0.007 compared with r-t-PA only; p = 0.009 compared with control peptide). (C) Annexin II was released from the cell surface by treatment with EGTA (50 mM) prior to incubation with r-t-PA with or without addition of peptide LCKLSL (5 mM). Data are expressed as the percentage of binding of control samples without competitor and represent the mean (SEM) of triplicate samples from a single experiment. Three experiments were performed with very similar results. Inset shows a western blot performed with supernatants concentrated from control cells and EGTA treated cells blotted with annexin II antibody. Statistical significance was determined by the Student's t test: *p = 0.009, **p = 0.0004, ***p = 0.0001.
sites other than PAI-1 can be analysed by evaluating the fraction of active enzyme bound to the cell surfaces. To analyse putative receptors for t-PA that maintain the protease proteolytically active in pancreatic cancer cells, we used rt-PA and measured the activity of the bound protease by its ability to activate plasmin and a fluorogenic plasmin substrate.

Firstly, we determined the linearity of plasmin generation by incubating PANC-1 cells with rt-PA (15 and 250 nM) over 20 minutes and, after washing out the unbound ligand, measuring hydrolysis of the AMC substrate at different times. As shown in fig 1A, generation of proteolysed AMC substrate is linear up to 30 minutes for t-PA at 15 nM ($r = 0.990$). When t-PA is used at 250 nM, plasmin generation is linear up to 20 minutes ($r = 0.983$). Therefore, all subsequent determinations of bound t-PA activity were performed after 15 minutes of incubation with substrate.

Next, we determined the time for t-PA binding to reach equilibrium. As shown in fig 1B, binding of t-PA to PANC-1 cells at 37°C was time dependent and reached equilibrium after 20 minutes. Using non-linear regression analyses, the data depicted in fig 1B fit to a monoexponential curve, suggesting a reversible bimolecular interaction.

We then determined the saturability of t-PA binding by incubating PANC-1 cells with increasing concentrations of rt-PA. As shown in fig 2A, cells demonstrated dose related and saturable binding of t-PA that reached a plateau at input doses of 100 nM. Non-linear regression analyses of these data showed that the best fit was to a one site binding model, with a $K_D$ of 42 nM. In parallel experiments, binding of t-PA to SK-PC-1 cells gave very similar results ($K_D$ 23.5 nM; fig 2B). These values are very similar to previously reported binding affinity values to purified annexin II ($K_D$ 25 nM$^{22}$), to 293 cells transfected with annexin II cDNA ($K_D$ 48 nM$^{37}$), or to an unidentified receptor molecule on vascular smooth muscle cells ($K_D$ 25 nM$^{30}$). The specificity of this binding was demonstrated by gradual inhibition of bound t-PA in cells treated with increasing concentrations of DFP inactivated...
Annexin II is a receptor for t-PA in pancreatic cancer cells

rt-PA; at 75 M excess of competitor t-PA, binding was reduced by 73.5% (fig 3A).

To discard the fact that the observed binding of t-PA involves interactions with acellular components, such as proteins of the extracellular matrix, cells in suspension were also assayed. Results from these experiments indicated saturable binding of t-PA to PANC-1 cells in suspension, with a *K*<sub>D</sub> of 34 nM, very similar to the results obtained previously (not shown). Thus pancreatic cancer cells express a surface protein capable of binding t-PA specifically and with high affinity while maintaining its activity, and thus may be the mediator of t-PA activities in these tumours.15

**t-PA interacts with annexin II on the surface of pancreatic cancer cells**

Because annexin II, the receptor for t-PA in endothelial cells, was previously determined to be overexpressed in pancreatic cancer,16–19 we studied its contribution to the binding of t-PA to pancreatic cancer cells. Earlier work identified the sequence LCKLSL (residues 8–13) from the annexin II protein as the critical region involved in the interaction with t-PA.20 This peptide inhibited 95% of binding of t-PA to annexin II.21 As shown in fig 3B, binding of rt-PA to PANC-1 cells was significantly reduced to mean 60 (SEM 3)% by peptide LCKLSL, but no inhibition was observed with the control peptide LGKLSL (binding was 99 (4)%), suggesting that annexin II may be involved in t-PA binding to pancreatic cells, and confirming that the Cys<sup>7</sup> of annexin II is required for the interaction.22 To prove these results, cells were treated with EGTA to reduce the amount of annexin II from the cell surface.23 In the presence of EGTA, annexin II was recovered in the supernatant (fig 3C, inset) and binding of rt-PA to cells was reduced to 65.9 (4.4)% (fig 3 C). However, when EGTA was added in combination with peptide LCKLSL, t-PA binding was further reduced to 36.8 (2.4)%. These results support the notion that the interaction with annexin II accounts for most of the t-PA binding to pancreatic cell surfaces.

To confirm the interaction of t-PA with annexin II, we performed co-immunoprecipitation experiments using specific antibodies on SK-PC-1 cells that express high levels of both proteins. Isotype matched antibodies to annexin I were used as a control. Immunoprecipitations with antiannexin II antibody followed by western blotting analyses with anti-t-PA antibody revealed the presence of t-PA in immunoprecipitated proteins (fig 4). In contrast, no detectable t-PA was present in immunocomplexes formed by annexin I antibodies. Conversely, when cell extracts were immunoprecipitated with anti-t-PA antibody, annexin II was co-immunoprecipitated. However, not all t-PA secreted by SK-PC-1 cells co-immunoprecipitated with annexin II, and vice versa, suggesting that they may correspond to molecular forms that do not interact with each other.

### t-PA and annexin II colocalise at the surface of human pancreatic tumours

To determine whether the interaction of t-PA and annexin II is present in vivo, we analysed the distribution pattern of annexin II and t-PA in human tumour tissue sections by double immunofluorescent staining and confocal microscopy. As shown in fig 5, both proteins were expressed at high levels in epithelial cancer cells and colocalisation was detected at membrane sites. t-PA and annexin II were also found at independent locations, in agreement with our previous results.

### Annexin II and t-PA overexpression correlate with invasion in pancreatic cancer cells

Annexin II overexpression in pancreatic, colon, and gastric cancer cells correlates with a poor prognosis.15,16–18 We studied expression of annexin II in several pancreatic cancer cell lines by western blotting and the invasion abilities of these cells in vitro, on Matrigel coated filters. As shown in fig 6, levels of expression of annexin II in several pancreatic cancer cell lines were normalised by subsequent incubation with antibodies to t-PA and B-actin. The histogram at the bottom shows the ratio of annexin II/t-PA values obtained by laser scanning densitometry of autoradiograms.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Expression of tissue-type plasminogen activator (t-PA) and annexin II, and invasive capacity of pancreatic cancer cells</th>
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<tbody>
<tr>
<td>Cell line</td>
<td>t-PA&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SK-PC-1</td>
<td>20</td>
</tr>
<tr>
<td>CAPAN-1</td>
<td>17.8</td>
</tr>
<tr>
<td>RWP-1</td>
<td>5.1</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>0</td>
</tr>
<tr>
<td>PANC-1</td>
<td>0</td>
</tr>
<tr>
<td>H766T</td>
<td>0</td>
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</tbody>
</table>

<sup>1</sup>Expression of t-PA, as determined by ELISA assays. 15 <sup>†</sup>Expression of annexin II determined by western blotting was normalised to expression of t-PA. <sup>‡</sup>The invasive capacity of cells in vitro was determined and quantified as described in experimental procedures.

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Figure 6  Expression of annexin II in pancreatic cancer cell lines. Western blotting with antiannexin II antibody of cellular protein extracts (50 µg) from the cell lines: lane 1, SK-PC-1; lane 2, CAPAN-1; lane 3, RWP-1; lane 4, BxPC-3; lane 5, PANC-1; and lane 6, H766T. Blots were normalised by subsequent incubation with antibodies to β-actin. The histogram at the bottom shows the ratio of annexin II/β-actin values obtained by laser scanning densitometry of autoradiograms.
DISCUSSION

We have previously shown that t-PA, highly expressed in the majority of pancreatic tumours and cell lines but not in normal pancreatic tissue, is required for the invasion, proliferation, and angiogenesis of these tumours. We have also shown that annexin II is overexpressed in pancreatic adenocarcinomas and localises at the basolateral compartment of SK-PC-1 cells. Localisation of active t-PA at the cell surface permits local activation of plasmin, imparts proteolytic and invasive advantages to tumour cells, and is also essential for t-PA dependent activation of signal transduction. However, so far the identities of the specific cell surface binding molecules for t-PA in pancreatic cancer cells have not been elucidated.

In the present study, we demonstrated the presence of high affinity binding sites on cell membranes that maintain t-PA as an active enzyme and contribute to the invasive properties of SK-PC-1 cancer cells. By measuring only active t-PA bound to extracellular membranes of cells, we have detected specific and saturable binding for t-PA in SK-PC-1 and PANC-1 cells. This binding was significantly inhibited by a specific peptide corresponding to the N terminus of the annexin II protein (residues 8–13), and also by treatment with EGTA that removes most of annexin II from the cell surface, suggesting that annexin II is a binding site for t-PA. In addition, the interaction was demonstrated by co-immunoprecipitation in cell extracts in vitro and co-immunolocalisation in tumours. However, so far the identities of the specific cell surface binding molecules for t-PA in pancreatic cancer cells have not been elucidated.

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In HUVEC cells, treatments with EGTA, antisense oligonucleotides to annexin II, or antiannexin II antibodies results in a reduction in specific binding of t-PA to 40–50% of controls, values comparable with our results in pancreatic cells obtained with EGTA or the peptide LCKLSE. Interestingly, the inhibitory effect on t-PA binding to pancreatic cancer cells after treatment with EGTA combined with the peptide LCKLSE was higher than inhibition from individual treatments. These results may be explained by the following: (i) annexin II may be present on surface membranes as monomers or heterotetramers with p11; (ii) in HUVEC cells, a portion of annexin II, likely the heterotetrameric annexin II/p11, binds to membranes in a Ca\(^{2+}\) independent way and is thus resistant to EGTA treatment; and (iii) the activity of t-PA is increased when it is bound to the heterotetrameric annexin II with respect to monomeric annexin II. Thus in our cells the efficacy of EGTA treatment on t-PA binding to annexin II may be partially occulted by the increased activity of t-PA bound to the heterotetrameric annexin II. In summary, inhibition of the interaction obtained by EGTA and the inhibitory peptide is significant, consistent with annexin II being a major binding site for t-PA on the surface of pancreatic tumour cells.

None the less, in polarised SK-PC-1 cells, t-PA is also bound to external apical membranes not containing annexin II (VD and RP, unpublished observations), suggesting that an additional protein(s) may function as a binding site for t-PA in these cells. In vascular smooth muscle cells, a high affinity binding site different from annexin II that maintains the protease active and shows a K\(_D\) (25 nM) very similar to that described here has recently been identified as the transmembrane protein CKAP4. Amphoterin, that binds t-PA in the central nervous system and is implicated in invasion, is also expressed by SK-PC-1 cells at high levels (VD and RP, unpublished observations). However, the non-saturable binding kinetics of t-PA to this site suggest that it is not the binding site detected in our experiments. Cytokeratin 8
also binds t-PA in a specific and saturable manner in breast cancer cells\(^3\) and promotes cell surface plasmin generation, but the reported \(K_d\) (250 (48) nM) is significantly different from that determined in our experiments.\(^2\) These observations suggest that pancreatic tumour cells may have more than one membrane molecule, with similar binding affinities for t-PA, possibly to focus plasmin generation at different membrane sites, the relevance of which in the process of tumour progression must be investigated.

Amphoterin, cytokeratin 8, and annexin II are cytotoxic proteins lacking a signal sequence, and yet extracellular functions for these proteins have been described. In this regard, annexin II has been found in exosomes, an unconventional secretion pathway for proteins lacking a signal sequence.\(^4\)

Our co-immunoprecipitation experiments clearly demonstrated the interaction of t-PA with annexin II. However, only a fraction of the two proteins interact with each other, likely corresponding to the fractions localized at the surface compartment of cells. The partial co-immunolocalisation of both proteins in vivo supports this notion, showing both t-PA and annexin II overexpression in tumours and colocalisation at particular membrane sites.

The LCKSL peptide inhibits t-PA binding to cells and invasion through Matrigel at comparable levels, confirming that the active t-PA bound to surface annexin II is needed for invasion. SK-PC-1 and PACN-1 cells also express high levels of u-PA and u-PAR that contribute to the invasion process.\(^13\) \(^15\) \(^47\) Thus inhibition of invasion obtained by the disruption of annexin II/t-PA interaction in the experiments shown here may account for most of the t-PA dependent invasive capacity.

Our results are the first to show the specific interaction of t-PA with annexin II on pancreatic cancer cell surfaces and its relevance for the invasive process. Understanding the precise mode of interaction of t-PA with pancreatic cancer cells and the molecular mechanisms used for invasion will help in the understanding of the contribution of this protease to cancer progression and in the development of more effective therapeutic strategies in the future.

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EDITOR’S QUIZ: GI SNAPSHOT

An unusual treatment for a colonic polyp

Robin Spiller, Editor

Clinical presentation
A 42 year old woman, gravida 3 para 2, was referred to our outpatient clinic for abdominal pain, bloody diarrhoea, and sideropenic anaemia (haemoglobin 9.9 g/dl, iron 40 µg/dl; ferritin 3 ng/dl). Eight months earlier she had undergone colonoscopy. A polyp in the sigmoid colon had been found and removed by hot snare polypectomy. Histological examination had showed the presence of endometrial foci. After polypectomy, symptoms disappeared. She had never complained of dysmenorrhoea or dyspareunia, symptoms often associated with endometriosis.

Six months later symptoms recurred. We performed colonoscopy twice, during menses and in the intermenstrual period (figs 1, 2, respectively). Colonoscopies showed a polypoid lesion at the same site of the previous polypectomy.

Question
What do these endoscopic images suggest and how would you manage this patient?
See page 1019 for answer

This case is submitted by:

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