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Differential endocytosis of tissue plasminogen activator by serpins PAI-1 and PAI-2 on human peripheral blood monocytes

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Abstract

Generation of the broad spectrum protease plasmin is facilitated by the tissue (t-PA) and urokinase (u-PA) plasminogen activators, within multiple physiological and disease states. Finely tuned control of this proteolytic cascade is exerted by the plasminogen activator inhibitors type-1 (PAI-1/SERPINE1) and 2 (PAI-2/SERPINB2). Expression of this network of activators and inhibitors by cells of myeloid lineage appears to be highly interchangeable between physiological environments, and whilst the role of PAI-1 and PAI-2 in regulating u-PA-dependent functions is well established, the interaction between t-PA and PAI-2 on these cell types is poorly characterised. To this end, we used freshly isolated peripheral blood monocytes (PBM) as a model of a t-PA-dependent cellular environment. We demonstrate that while both PAI-1 and PAI-2 could inhibit surface-bound t-PA and are internalised predominately via low-density-lipoprotein receptor family members, PAI-1 enhanced the endocytosis of t-PA, whereas PAI-2 did not. Surface plasmon resonance analyses revealed differential binding affinities between the very-low-density-lipoprotein receptor and t-PA and t-PA:PAI-1 complexes in addition to those previously described with low-density-lipoprotein receptor-related protein. Moreover, t-PA:PAI-2 bound to both endocytosis receptors with similar kinetics to t-PA. These differential biochemical interactions between t-PA and the t-PA:PAI complexes may underlie the observed differences in endocytosis mechanisms on the PBMs. This suggests that while PAI-1 and PAI-2 function similarly in the control of cellular plasmin generation by t-PA, they may have disparate effects on the alternative functions of t-PA via modulation of its engagement with endocytosis receptors.

Keywords

differential, endocytosis, tissue, plasminogen, activator, human, serpins, 2, monocytes, blood, 1, peripheral, pai, CMMB

Disciplines

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Differential endocytosis of tissue plasminogen activator by serpins PAI-1 and PAI-2 on human peripheral blood monocytes

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Summary

Generation of the broad spectrum protease plasmin is facilitated by the tissue (t-PA) and urokinase (u-PA) plasminogen activators, within multiple physiological and disease states. Finely tuned control of this proteolytic cascade is exerted by the plasminogen activator inhibitors type-1 (PAI-1/SERPINE1) and 2 (PAI-2/SERPINB2). Expression of this network of activators and inhibitors by cells of myeloid lineage appears to be highly interchangeable between physiological environments, and whilst the role of PAI-1 and PAI-2 in regulating u-PA-dependent functions is well established, the interaction between t-PA and PAI-2 on these cell types is poorly characterised. To this end, we used freshly isolated peripheral blood monocytes (PBM) as a model of a t-PA-dependent cellular environment. We demonstrate that while both PAI-1 and PAI-2 could inhibit surface-bound t-PA and are internalised predominantly via low-density-lipoprotein receptor family members, PAI-1 enhanced the endocytosis of t-PA, whereas PAI-2 did not. Surface plasmon resonance analyses revealed differential binding affinities be-

tween the very-low-density-lipoprotein receptor and t-PA and t-PA:PAI-1 complexes in addition to those previously described with low-density-lipoprotein receptor-related protein. Moreover, t-PA:PAI-2 bound to both endocytosis receptors with similar kinetics to t-PA. These differential biochemical interactions between t-PA and the t-PA:PAI complexes may underlie the observed differences in endocytosis mechanisms on the PBMs. This suggests that while PAI-1 and PAI-2 function similarly in the control of cellular plasmin generation by t-PA, they may have disparate effects on the alternative functions of t-PA via modulation of its engagement with endocytosis receptors.

Keywords

Cell surface protein, endocytosis, protein-protein interactions, serpin, tissue plasminogen activator (t-PA), peripheral blood monocytes, plasminogen activator inhibitor type-1, plasminogen activator inhibitor type-2

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Introduction

The tissue (t-PA) and urokinase (u-PA) plasminogen activators convert plasminogen to plasmin, which is involved in fibrinolysis, tissue remodelling and cell migration. u-PA is associated with proteolysis within the pericellular environment; whilst the role of t-PA has been coupled with fibrinolysis (1). More recent reports suggest that t-PA is not restricted to maintaining homeostasis of coagulation and fibrinolysis. For example, t-PA has been shown to play a role in angiogenesis and neurobiology via cell surface interactions with annexin II (p36), the annexin II heterotetramer (AIIt) (comprised of two subunits of p36 and two of p11) and the low-density-lipoprotein-receptor-related protein (LRP) (2–4). Furthermore, cells of myeloid lineage interchangeably express t-PA, u-PA and their specific inhibitors specifically during activation or differentiation processes (5–6), though the physiological implications of this requires further investigation.

t-PA is a 72 kDa glycosylated protein, which is secreted in a single chain form (sc-t-PA) by peripheral blood monocytes (7) and endothelial cells (8). Cleavage of sc-t-PA produces the 62.9 kDa two chain (tc) t-PA conformation (9). Both sc-t-PA and tc-t-PA display enzymatic activity towards plasminogen, which is enhanced when they are bound to fibrin (10). In addition to fibrin interactions, both forms of t-PA also bind, via lysine dependent interactions, to monocyte, macrophage, endothelial and human tumour cell lines and efficiently activate cell-bound plasminogen (11–14).

Control of plasmin generation by sc-t-PA or tc-t-PA *in vivo* is thought to be mediated predominantly through the inhibitory functions of the plasminogen activator inhibitor type 1 (PAI-1/SERPINE1) and potentially to a lesser extent by plasminogen activator inhibitor type 2 (PAI-2/SERPINB2) (15). As members of the serine protease inhibitor (serpin) family, PAI-1 and PAI-2 exhibit a suicide substrate-like inhibitory mechanism via the

formation of a 1:1 covalently linked, serpin-protease complex (16–17). Both PAI-1 and PAI-2 efficiently inhibit tc-t-PA in solution phase with second order rate constants of $\sim 10^7$ and 10^5 $\text{M}^{-1}\cdot\text{s}^{-1}$, respectively, and both are inefficient inhibitors of fibrin bound t-PA (18, 19). Therefore t-PA:PAI complexes are typically formed after t-PA dissociates from the fibrin clot. However, PAI-1 is considered the primary inhibitor of t-PA as it is able to form serpin-protease complexes with both sc-t-PA and tc-t-PA and is found in normal human plasma at concentrations ranging from 140 pM to 1.9 nM (20). PAI-2 does not interact with sc-t-PA in plasma and is only detected during late stage pregnancy (20) but, with concentrations reaching approximately 200 $\text{ng}\cdot\text{mL}^{-1}$ (4 nM), PAI-2 has been proposed to play a role in maintaining placental integrity by moderating excessive fibrinolysis in normal pregnancy (21). Despite this, little is understood about the role of PAI-2 as a physiological inhibitor of t-PA due to limited reports of t-PA:PAI-2 complexes identified *in vivo* (1). Yet, co-expression of t-PA and PAI-2 has been shown in normal bone marrow (5), skin (22), saliva and salivary gland tissue (23), gingival fluid (24–25), as well during pregnancy (21), and PAI-2 is able to inhibit cell surface bound tc-t-PA *in vitro* (13). Furthermore, enhanced expression of both t-PA and PAI-2 by epithelial cells is indicative of some inflammatory disease states, such as periodontal disease (24) and psoriasis (1), strongly suggesting a role for PAI-2 in the control of t-PA activity at sites of localised inflammation. This is further supported by the finding that PAI-2 but not PAI-1 is relatively resistant to oxidative conditions which are symptomatic of inflammation (26).

Whilst PAI-2 expression can be up-regulated by a variety of cell types under stress conditions, the predominant physiological producers of PAI-2 are monocytes and macrophages (1). Monocytes also express and secrete t-PA (7) which has been shown to bind to the surface of these cells via numerous and varied receptors (11, 12, 27). Furthermore, Ritchie et al. (28) reported the up-regulation of PAI-2 expression by monocytes cultured in the presence of thrombin. This suggests a potential role for PAI-2 in the homeostatic balance between thrombosis and fibrinolysis at local sites of inflammation or during wound healing processes, possibly through the inhibition and clearance of tc-t-PA from the pericellular environment.

Clearance of t-PA and t-PA:PAI-1 from the circulation via endocytic processes in the liver is well characterised (29, 30). The mechanisms for endocytosis of these ligands have been shown to be dependent on members of the low-density-lipoprotein receptor (LDLR) family, specifically LRP (31, 32) and scavenger receptors such as the mannose receptor (CD206) (27). The rate of endocytosis of PAI-1-inhibited t-PA was shown to be accelerated compared to t-PA only, on rat hepatoma cells (31). This acceleration in clearance was found to be due to the increased affinity of t-PA:PAI-1 complexes for LRP (32). Interestingly, Camani et al. (33) found that Novikoff rat hepatoma cells, MEF-1 mouse embryonic fibroblasts, HT1080 fibroblasts and COS African green monkey kidney cells were all able to degrade $[^{125}\text{I}]$ -t-PA and $[^{125}\text{I}]$ -t-PA:PAI-1 via LRP-mediated uptake, whilst the THP-1 and U-937 monocytic cell lines were not. The authors suggested that a co-receptor, absent on the THP-1 and U-937 cell lines, may be required for these ligands

to interact with LDLRs. However, t-PA was shown to specifically bind to the surface of freshly isolated peripheral blood monocytes (PBM), THP-1 and U-937 cell lines (11). Macrophages derived from human PBMs were shown to take up and degrade $[^{125}\text{I}]$ -t-PA via the mannose receptor (12). Furthermore, Simon et al. (34) showed LDLR-family dependent endocytosis and degradation of $[^{125}\text{I}]$ -t-PA:PAI-1 by the THP-1 cell line. Thus, the potential function of PAI-1 or PAI-2 in the inhibition and enhanced endocytosis of t-PA bound to myeloid cell types remains unclear.

In the present study, we aimed to clarify and compare the role of these serpins as regulators of cellular t-PA and the fate of t-PA:serpin inhibitory complexes. Freshly isolated PBMs were utilised as a non-transformed t-PA-dependent cellular model representing myeloid lineages (5). These were profiled for known t-PA receptors as well as endocytosis receptors associated with protease:serpin complex cell surface clearance, such as LRP and the very-low-density-lipoprotein receptor (VLDLR). In addition, as the interactions of t-PA:PAI-2 with LRP, or t-PA and t-PA:PAI complexes with VLDLR had not previously been studied, surface plasmon resonance (SPR) was used to compare the binding kinetics of all three ligands to both these receptors.

Methods

Materials

Recombinant human PAI-2 (47 kDa form) was provided by PAI-2 Pty Ltd (Sydney, Australia). Purified human receptor associated protein (RAP), recombinant human PAI-1 (stable mutant) and human tc-t-PA were obtained from Molecular Innovations (Novi, MI, USA). Spectrozyme PL substrate, murine anti-human u-PA B-chain IgG₁ (#394), murine anti-human t-PA IgG₁ (#E-4) and murine anti-human LRP α -chain IgG₁ (#3402) were supplied by American Diagnostica Inc. (Stamford, CT, USA). Rabbit anti-human VLDLR IgG and rabbit anti-annexin II (p36) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-S100A10 (p11) was from R&D Systems (Indianapolis, IN, USA). Goat anti-rabbit FITC conjugate IgG (whole molecule), goat anti-mouse IgG (Fab specific) FITC conjugate, rabbit anti-goat IgG (whole molecule) FITC conjugate, goat IgG, propidium iodide (PI), Hanks balanced salt modified and non-modified, bovine serum albumin (BSA), human fibrin, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) and OptiPrep™ density gradient medium were from Sigma-Aldrich (St. Louis, MO, USA). Alexa 488 protein labelling kit and Alexa 488 quenching polyclonal antibody were from Molecular Probes (Eugene, OR, USA). Murine α -CD14-texas red conjugated monoclonal antibody was purchased from Invitrogen (Carlsbad, CA, USA). Murine α -CD206 monoclonal antibody was purchased from Hycult-biotech (Uden, The Netherlands). Recombinant human t-PA (Actylise, a mixture of sc and tc-t-PA) was a kind gift from Boehringer-Ingelheim (North Ryde, NSW, Australia).

Isolation of human PBMs

Peripheral venous blood was collected from donors into EDTA vacuum tubes (BD Biosciences, North Ryde, NSW, Australia) under controlled conditions by a trained phlebotomist at the University of Wollongong (UOW) (protocol approved by UOW, Human Ethics Committee, approval number HE00/221). Buffy coats were prepared by centrifuging whole blood samples at 1,300 g for 30 minutes (min). White blood cells were removed under sterile conditions and monocytes isolated using OptiPrep™ as per the manufacturer's instructions. This methodology yielded cell preparations containing 60 to 80% CD14 positive (monocyte) cells as determined by flow cytometry. Freshly isolated PBMs were used immediately for all experiments.

Plasminogen activation assays

Cells were washed and resuspended at 1×10^6 cells.mL⁻¹ in Tris Buffered Saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4), incubated on ice in the absence or presence of 100 nM t-PA for 40 min and then washed twice in TBS to remove excess unbound protein. Triplicate 50 µl cell suspensions were then aliquoted into 96-well microtitre plates and incubated for 15 min at room temperature, in the absence or presence of increasing concentrations of PAI-1, PAI-2 and 10 µg.mL⁻¹ plasminogen made up to a final volume of 100 µl with TBS buffer. Spectrozyme PL substrate (100 µl) was then added to a final concentration of 0.25 mM and substrate conversion by plasmin measured kinetically at 405 nm over 2 hours at 37°C using a SpectraMax 250 plate reader; data was recorded using SoftMax® Pro software (Molecular Devices). Negative controls contained cells alone (no t-PA or plasminogen). Other controls consisted of t-PA (100 nM) in the absence or presence of 1.4 µM fibrin, plasminogen (10 µg.mL⁻¹) and Spectrozyme PL (0.25 mM), as previously described (13). Background absorbance (buffer only wells) was subtracted from all data.

Flow cytometry analysis

Cell surface antigen detection

Membrane bound antigens were detected using indirect immunofluorescence assays, essentially as previously described (35). Briefly, cells were suspended at a final concentration of 1×10^6 cells.mL⁻¹ in ice cold binding buffer (phenol red-free Hanks buffered salt solution, pH 7.4, containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.1% BSA) containing 10 µg.mL⁻¹ of primary antibody and incubated on ice for 40 min. After washing steps, the cells were resuspended with ice-cold binding buffer containing 1:100 dilution of FITC-conjugated secondary antibody, followed by incubation with 10 µg.mL⁻¹ α-CD14-texas red conjugated antibody (for identification of PBMs). Cells were then washed prior to analysis by flow cytometry, with viable cell populations selected through

propidium iodide (PI) exclusion. Isotype matched control antibodies were used to assess non-specific binding due to the primary antibody. All data obtained was analysed using FLOJO software version 7.1 (Treestar, Inc, Ashland, OR, USA) and was restricted to CD14-positive/PI-negative cells. All geometric mean fluorescence values reported represent mean fluorescence intensity for specific binding ± SEM. All assays were conducted in triplicate.

Protein binding assays

Cell surface binding analyses were conducted by incubating cells on ice in binding buffer containing increasing amounts of Alexa488-labelled t-PA (t-PA_{Alexa488}) or BSA (BSA_{Alexa488}) (negative control) for 40 min. This was done in order to allow maximal cell surface binding by also minimising internalisation of t-PA. Cells were then washed twice with ice cold PBS and analysed using flow cytometry as described above.

Internalisation assays

These were conducted as previously described (36). Briefly, cells were incubated for 40 min at 1×10^6 cells.mL⁻¹ in binding buffer, containing 0 (control) or 100 nM t-PA or t-PA_{Alexa488}, on ice to minimise internalisation. After washing to remove excess unbound t-PA, cells were incubated with either PAI-2 or PAI-2_{Alexa488} (where stated), PAI-1 or no additions and incubated at 37°C for the time periods indicated. All subsequent steps were performed at 4°C. The cells were next washed twice and incubated for 30 min with 4 µg.mL⁻¹ alexa488 quenching antibody (quenches any remaining cell surface fluorescence and is thus a measure of internalised ligand). Then, after washing, the cells were incubated for 40 min with 10 µg.mL⁻¹ α-CD14 monoclonal antibody. Finally, the cells were washed twice with ice cold PBS and analysed using flow cytometry as described above.

Surface plasmon resonance assays

Kinetic analysis was performed using a BIAcore 2000 (BIAcore AB, Uppsala, Sweden). LRP or VLDLR was immobilised on to a CM5 BIAcore chip according to the manufacturer's instructions. Briefly, the chip was activated using a 1:1 mixture of 0.2 M *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodi-imide and 0.05 M *N*-hydroxysuccinimide. LRP or VLDLR was coated on to the chip at 40 µg.mL⁻¹ in 10 mM sodium acetate buffer (pH 3) for 7 min at 5 µl.min⁻¹, as previously described (37), to a level of 2,000 response units (VLDLR) or 2,000 to 10,000 response units (LRP). The unoccupied binding sites were blocked using 1 M ethanolamine, pH 8.5. Analytes (t-PA, t-PA:PAI) were buffer exchanged into running buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 1 mM CaCl₂, 0.05% v/v Tween-20) before applying to the BIAcore chip at 20 µl.min⁻¹. A serial dilution of analytes ranging from 500 – 15 nM were used to allow kinetic analyses. t-PA:PAI complexes were prepared by incubating t-PA with PAI-1 (1:1 molar ratio) or PAI-2

Table 1: Ligand expression profiles of plasminogen activators and associated receptors.

| | Peripheral blood monocytes |
|--------------------------|----------------------------|
| Ligand | Relative MFI \pm SEM |
| u-PA | 1.82 ± 0.5 |
| t-PA | 5 ± 0.9 |
| LRP | 15.7 ± 1.3 |
| VLDLR | 70.5 ± 0.8 |
| Annexin 2A (P36) | 68.78 ± 5.9 |
| S100A10 (P11) | ND |
| Mannose receptor (CD206) | ND |

Data represents mean fluorescence intensity (MFI), with relevant matched isotype control values subtracted ($n=3$, \pm SEM). ND, not detected.

(1:3 molar ratio) for 60 min at 37°C. An excess of PAI-2 could be used and not removed from the t-PA:PAI-2 solutions as un-reacted PAI-2 does not bind LRP or VLDLR (36–38). Non-reducing SDS-PAGE confirmed the presence of t-PA:PAI-1 or t-PA:PAI-2 complexes with minimal/negligible residual free t-PA or PAI-1 (data not shown). In all cases the analyte concentration refers to the concentration of t-PA present. Regeneration of the chip was achieved using 100 mM H_3PSO_4 . All buffers were filtered and degassed before use. For kinetic analysis, a blank cell was used as the reference cell and data was analysed using BIAevaluation software (Version 3).

Statistical analysis

Statistical significance was determined using Students t-test and was performed using GraphPad Prism version 5.00 for Windows,

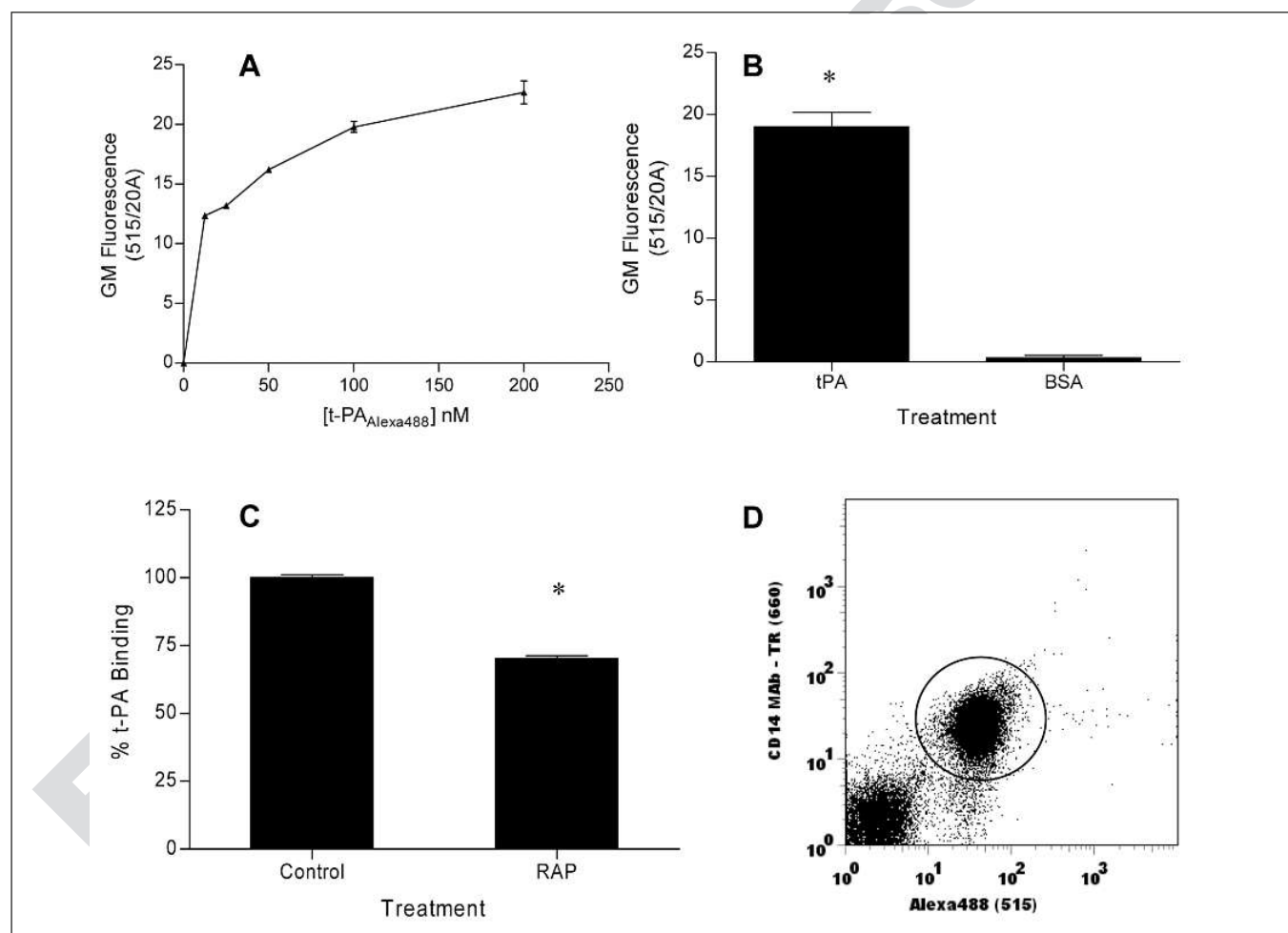


Figure 1: Characterisation of t-PA binding to PBMs. A) Plot showing the concentration dependence of the binding of t-PA_{Alexa488} to the surface of PBMs. B) Binding of 200 nM t-PA_{Alexa488} or BSA_{Alexa488} (control) to the cell surface of PBM. * $p < 0.05$ significantly different from t-PA. C) Plot showing the effects of pre-incubation with 200 nM RAP on cell surface binding of t-PA_{Al-}

exa488 using flow cytometry. * $p < 0.05$ significantly different from control (minus RAP). D) Two dimensional flow cytometry dot plot showing t-PA_{Al-}exa488 binding (515 nm) to CD14 positive (660 nm) cell population (circled). With the exception of panel D, data represents the geometric mean (GM) fluorescence with background (auto-fluorescence) subtracted, $n=3 \pm$ SEM.

GraphPad Software (San Diego, CA, USA; www.graphpad.com).

Results

To assess basal endogenous cell surface expression levels of plasminogen activators and endocytosis receptors of the LDLR family, immunofluorescence assays of PBMs were undertaken using flow cytometry. The PBMs expressed relatively low levels of u-PA and t-PA (► Table 1). LRP and VLDLR were also detected on PBMs (Table 1). We next investigated cell surface expression of the mannose receptor and AII_t (sub-units p36 and p11), both known cell surface receptors for t-PA (12, 40). Whilst p36 was expressed on the PBMs, neither the mannose receptor nor p11 were detectable on these cells (Table 1).

Since at least one known t-PA receptor, p36, was detected, we tested the binding of exogenous t-PA to PBMs. When incubated on ice to minimise internalisation and/or proteolytic activity, the binding of t-PA to PBMs was saturable within 40 min, showing maximum binding at about 200 nM (► Fig. 1A). A negative control BSA_{Alexa488} did not bind to the cell surface at the highest equivalent t-PA concentration (Fig. 1B). We next tested the contribution of LDLRs to the direct binding of t-PA to PBMs. Pre-incubation of the cells with RAP, a commonly used antagonist of ligand binding specific to the LDLR family, significantly reduced cell surface binding of exogenous t-PA to PBMs by 30% (Fig. 1C). This level of inhibition was consistent with previous studies using human umbilical vein endothelial cells (39), indicating that LDLR family members are functional cell surface receptors for t-PA in addition to the potentially numerous other receptors represented by the non-RAP inhibitable binding interactions. Furthermore, t-PA bound only to the CD14-positive stained (i.e. PBM) cells within the cell suspension, indicating that the other blood cell types present did not bind measurable (and therefore did not internalise) amounts of t-PA (Fig. 1D).

Saturation of the cell surface with exogenous t-PA caused a significant increase in cellular plasmin activity (► Fig. 2), which was only four-fold slower than t-PA mediated plasminogen activation in the presence of fibrin ($\Delta \text{O.D. min}^{-1} = 0.012 \pm 0.0001 \text{ SEM}$). The very low levels of intrinsic plasmin generation in the absence of exogenous t-PA confirmed the low expression of cell surface t-PA (or u-PA) as determined by immunofluorescence assays (Table 1). As expected from previous work showing inhibition of cell surface t-PA by PAI-2 on the MCF-7 and HeLa cell lines (13), PAI-2 was also able to inhibit cell surface t-PA on PBMs at similar concentrations (Fig. 2). PAI-1 was overall more efficient at inhibiting plasmin formation compared to PAI-2, resulting in 95–100% inhibition at all concentrations used (Fig. 2). However, at 50 nM PAI-2 inhibited about 80% of plasmin formation while at 200 nM both serpins inhibited plasmin formation by > 90%.

The fate of active and inactivated t-PA on PBMs was next examined. Freshly isolated PBMs were found to endocytose t-PA in the absence or presence of either serpin (► Fig. 3). However, PAI-1 significantly enhanced the proportion of t-PA internalised from the

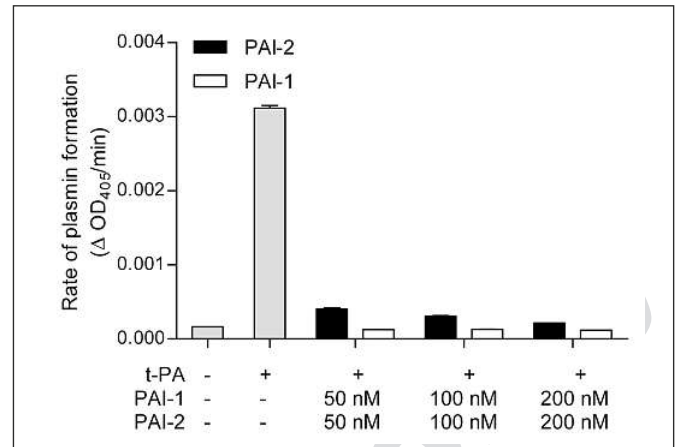


Figure 2: Plot showing inhibition of PBM-mediated plasminogen activation by exogenous PAI-1 and PAI-2. Freshly isolated PBMs were pre-incubated with or without 100 nM t-PA where indicated. Then the rate of plasmin formation from plasminogen in the absence or presence of PAI-2 or PAI-1 at concentrations indicated was measured as described in *Materials and methods*. Data shown represents mean \pm SEM, (n=3), with the background (cells only) value subtracted.

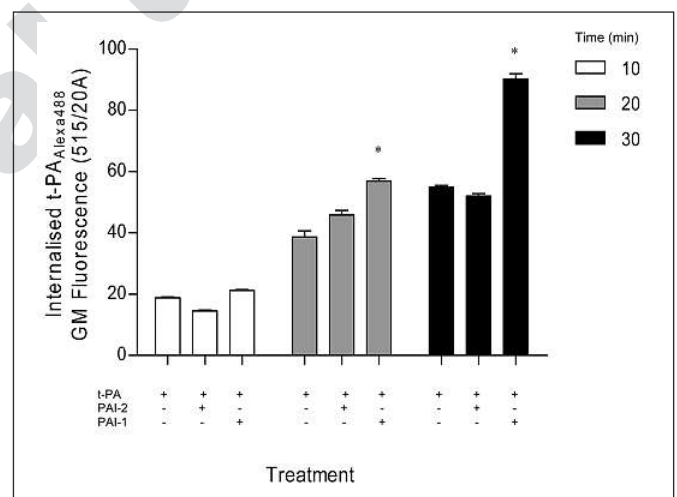


Figure 3: Plot showing PBM-mediated internalisation of t-PA by exogenous PAI-1 and PAI-2. Freshly isolated PBMs were pre-incubated with 100 nM t-PA_{Alexa488}. After washing, internalisation was assessed in the presence or absence of 100 nM PAI-1 or PAI-2 for the time periods indicated. Data represents the geometric mean (GM) fluorescence with background (auto-fluorescence) subtracted, n=3, \pm SEM. * $p < 0.05$ significantly different from t-PA only controls at each time point.

cell surface after 20 min and 30 min incubations whilst PAI-2 did not (Fig. 3). This differential effect on t-PA endocytosis by the serpins was surprising as PAI-2 has previously been shown to enhance the uptake of u-PA from the surface of prostate cancer cells, albeit not to the same extent as PAI-1 (37).

To ensure that PAI-2 not only inhibited (i.e. bound) to cell surface t-PA but was itself subsequently endocytosed as a t-PA:PAI-2

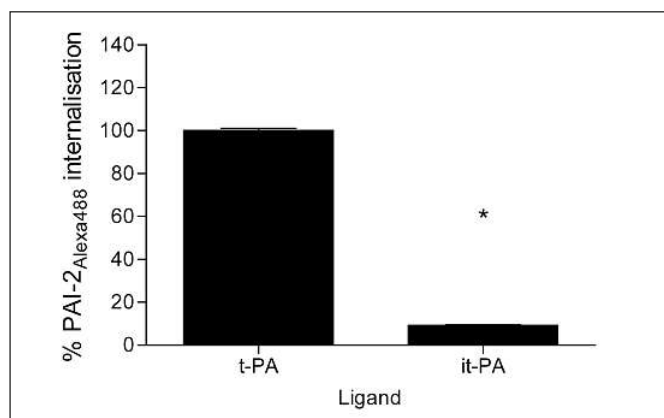


Figure 4: Plot showing t-PA dependent internalisation of PAI-2 by PBMs. PBMs were pre-incubated in the absence (no t-PA) or presence of either 100 nM t-PA (control) or PPACK inhibited t-PA (referred to as it-PA). Then internalisation of 10 nM PAI-2_{Alexa488} was determined, as described in *Materials and methods*. Data represents geometric mean (GM) fluorescence with background (auto-fluorescence) removed normalised (%) to control, $n=3$, \pm SEM. * $p<0.05$ significantly different from control.

complex, internalisation assays using PAI-2_{Alexa488} and exogenous active-t-PA or PPACK-inactivated t-PA were performed. Cell surface bound PPACK-inactivated t-PA significantly reduced the internalisation of PAI-2 by 90% (► Fig. 4), indicating that the majority of PAI-2 bound at the PBM cell surface was subsequently endocytosed as a t-PA:PAI-2 complex. This was also supported by the inability of PAI-2 to be internalised by cells not pre-incubated with exogenous t-PA (data not shown), which again confirmed that the levels of endogenous t-PA (or u-PA) on the PBMs were very low.

In order to confirm that endocytosis receptors are involved in the clearance of t-PA and t-PA:PAI complexes from the PBM cell surface, the roles of scavenger receptors and the LDLR family were investigated. RAP significantly inhibited t-PA:PAI complex internalisation by 80–90% in all cases (► Fig. 5). Fucoidin, which is

known to block scavenger receptors, inhibited only 10–20% of the internalisation (Fig. 5). This indicates that receptors of the LDLR family play a major role in clearing t-PA and t-PA:PAI complexes from the PBM cell surface, while scavenger receptors play only a minor role in this process. Previous studies identified the mannose receptor (CD206) as a t-PA clearance receptor on liver cells (40) and expression of this receptor has been reported for primary macrophages (12). However, as PBMs did not express detectable levels of the mannose receptor (Table 1), it is unlikely that this receptor plays a role in the fucoidin-inhibitable clearance of t-PA or t-PA:PAI complexes by these cells. The identity of the fucoidin-inhibitable receptors that mediate a minor fraction of the clearance of t-PA and t-PA:PAI complexes from the surface of PBMs remains unclear.

Since the most likely endocytosis receptors on the PBMs were LRP and VLDLR (as ascertained by cell profiling; refer to Table 1), the kinetics of binding of t-PA and t-PA:PAI complexes to immobilised LRP and VLDLR were analysed using surface plasmon resonance (SPR). Initial specificity analyses using overlay plots of sensorgrams indicated that t-PA:PAI-1 bound more strongly to LRP than t-PA alone or in complex with PAI-2 (► Fig. 6A). This effect was less obvious with VLDLR (Fig. 6B). For kinetic analyses a serial dilution of ligands were used, which revealed dose dependent binding for all of the analytes (data not shown) and complex binding interactions of t-PA:PAI-1 to both LRP and VLDLR. Nevertheless, in both cases, the data best fit competitive heterogeneous analyte models, suggesting the presence of a relatively low- and a high-affinity binding site within the complex for both LRP ($K_{D1} \sim 21.2$ nM, $K_{D2} \sim 0.95$ nM, respectively) and VLDLR ($K_{D1} \sim 139$ nM; $K_{D2} \sim 23$ nM) (► Table 2). t-PA and t-PA:PAI-2 also bound to LRP and VLDLR. However, these data were best fit by a one-binding site model with t-PA and t-PA:PAI-2 binding with very similar moderate affinities to LRP (K_D 66–67 nM) and VLDLR (K_D 124–131 nM). Interestingly, the affinities of t-PA and t-PA:PAI-2 for each of these receptors was similar to that of the lower affinity binding interaction between t-PA:PAI-1 and LRP or VLDLR (Table 2). The

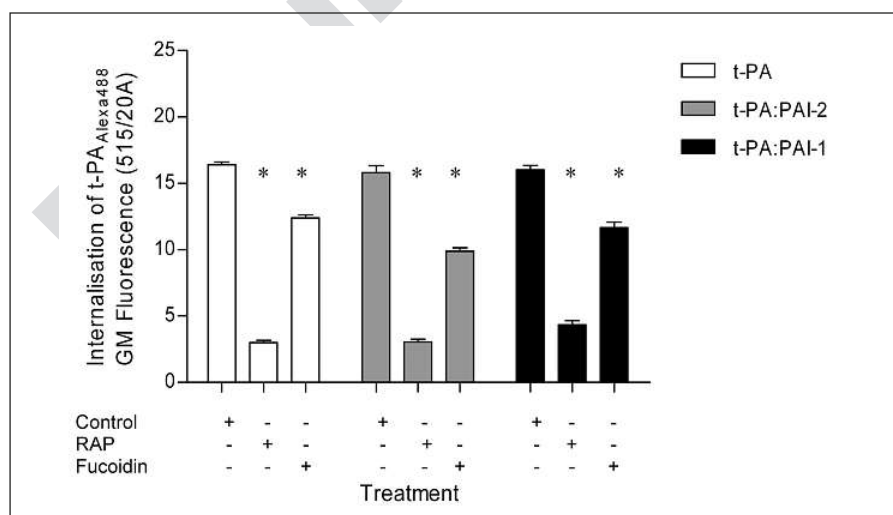


Figure 5: Identification of predominant endocytosis pathways. Internalisation of t-PA_{Alexa488} and t-PA_{Alexa488}:PAI complexes was measured in the absence (control) or presence of treatments as indicated below the plots. Data represents geometric mean (GM) fluorescence with background (auto-fluorescence) subtracted, $n=3$, \pm SEM. * $p<0.05$ significantly different from control.

higher affinity binding (K_{D2}) of t-PA:PAI-1 to both LRP and VLDLR was due to a substantial (100- to 16-fold, respectively) decrease in the dissociation rate constant, indicating much slower dissociation of the complex. The affinity of binding of t-PA and t-PA:PAI complexes to LRP was generally higher than that obtained with VLDLR (Table 2). To our knowledge, there is no previously published data describing the binding of t-PA:PAI-2 to LRP and VLDLR but the affinities reported here for the binding of t-PA and t-PA:PAI-1 to LRP are comparable to those obtained by others using SPR (43).

Discussion

The results presented here show that both PAI-2 and PAI-1 inhibit the enzymatic activity of PBM cell surface bound t-PA and that t-PA:PAI complexes formed at the cell surface are subsequently endocytosed, predominantly via an LDLR dependent pathway. However, the formation of a serpin-protease complex between t-PA and PAI-1, but not PAI-2, accelerates t-PA endocytosis. The differences in endocytosis of these ligands may be explained by the kinetic data obtained for the binding of t-PA and t-PA:PAI to LRP and VLDLR using SPR. The formation of complexes between PAI-1 and either t-PA or u-PA are thought to cause a conformational change within PAI-1 resulting in it exposing a high affinity binding site for LRP (32, 41) or potentially VLDLR, which contributes directly to the enhanced endocytosis of t-PA:PAI-1 or u-PA:PAI-1 complexes from the cell surface.

Both LRP (3, 38) and VLDLR (3, 37) play a pivotal role in the rapid clearance of surface bound u-PA:PAI-1 and u-PA:PAI-2 complexes. Like PAI-1, PAI-2 has been shown to accelerate u-PA internalisation due to an increased affinity of u-PA:PAI-2 for LRP and VLDLR compared to u-PA alone (38). It is possible that formation of the u-PA:PAI-2 complex results in a conformational change within u-PA as PAI-2 does not bind to either LRP or VLDLR (36–37). Importantly, t-PA alone and t-PA:PAI-2 show

identical one-site binding affinities for LRP and VLDLR, suggesting that complex formation does not alter the binding interaction of t-PA to LRP or VLDLR. Furthermore, especially in regards to VLDLR binding, the similar K_D values obtained for the lower affinity binding interactions of t-PA:PAI-1 compared to those observed for t-PA or t-PA:PAI-2 (Table 2) suggests that no functional con-

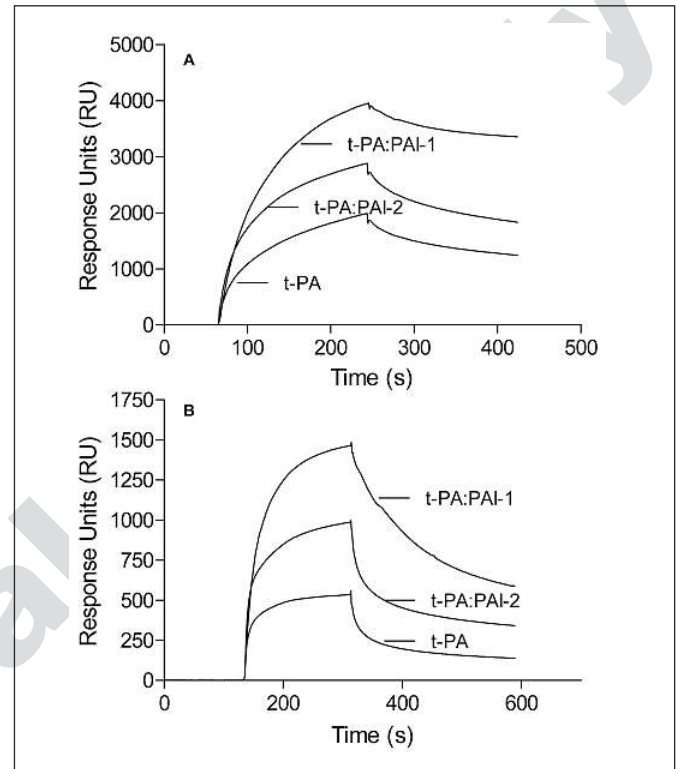


Figure 6: Surface plasmon resonance. Analysis of the interaction between tc-t-PA, tc-t-PA:PAI-1 or tc-t-PA:PAI-2 and LRP (A) or VLDLR (B). Sensorgrams show the interaction of the analytes (all at 250 nM) with immobilised LRP or VLDLR as the ligands. The data shown is representative of at least three independent experiments.

Table 2: Surface plasmon resonance kinetic analysis of the interaction between LDLRs and t-PA or t-PA:PAI complexes.

| LRP | | | | | |
|--|-----------------------------------|------------------------|-------------------------|---------|----------------|
| Analyte | Model | ka (1/Ms) | kd (1/s) | KD (nM) | χ ² |
| t-PA | 1:1 | 4.22 x 10 ⁵ | 0.0282 | 66.9 | 1.82 |
| t-PA:PAI-2 | 1:1 | 3.71 x 10 ⁵ | 0.0245 | 66.1 | 1.92 |
| t-PA:PAI-1 | Heterogeneous analyte – competing | 2.21 x 10 ⁵ | 4.69 x 10 ⁻³ | 21.2 | 5.61 |
| | | 4.62 x 10 ⁴ | 4.38 x 10 ⁻⁵ | 0.95 | |
| VLDLR | | | | | |
| t-PA | 1:1 | 4.18 x 10 ⁵ | 0.0517 | 124 | 2.55 |
| t-PA:PAI-2 | 1:1 | 4.16 x 10 ⁵ | 0.0544 | 131 | 2.28 |
| t-PA:PAI-1 | Heterogeneous analyte – competing | 5.37 x 10 ⁵ | 0.0748 | 139 | 5.07 |
| | | 2.00 x 10 ⁵ | 4.60 x 10 ⁻³ | 23.0 | |
| Binding data was fitted using the BIAevaluation 3.0 software. The binding model chosen represents that with the lowest χ ² value. | | | | | |

Binding data was fitted using the BIAevaluation 3.0 software. The binding model chosen represents that with the lowest χ^2 value.

formational change occurs within the t-PA molecule upon complex formation. Therefore it is likely that the differences in K_D values for LRP and VLDLR observed between t-PA:PAI-1 and t-PA or t-PA:PAI-2 are related to exposure of the high affinity cryptic binding site within PAI-1. Of note, comparison of the binding affinities of t-PA and t-PA:PAI for LRP and VLDLR reveals a preference of these ligands for LRP (► Table 3). In contrast, u-PA and u-PA:PAI appear to bind with higher affinities to VLDLR compared to LRP (Table 3). However, the significance of these differences is unclear at this stage.

Interestingly, RAP inhibited the direct binding of t-PA to the PBM cell surface by about 30%. This observation is similar to that reported by Mulder et al. (39), where pre-treatment with RAP resulted in a 25% reduction of t-PA binding to the cell surface of HU-VECs. The identities of other receptors responsible for the remaining proportion of t-PA binding are potentially numerous, of which p36 may partially contribute to total t-PA binding on these cells, as previously published (42). The absence of the mannose receptor on our freshly isolated PBMs cells was not surprising as it is indicative of a monocyte versus a differentiated macrophage phenotype (12). That fucoidin inhibited the clearance of t-PA and t-PA:PAI complexes from the surface of PBMs by about 10–20%, suggests that other scavenger receptors present on PBMs play a minor role in the binding and subsequent endocytosis of these ligands.

The traditional role of t-PA in fibrinolysis and of PAI-1 as a regulator of t-PA is well characterised. However, in recent years it has become increasingly clear that t-PA is not restricted to maintaining homeostasis of coagulation and fibrinolysis within the vasculature. t-PA has been found to induce matrix metalloproteinase-9 gene expression in rat kidney interstitial fibroblast and promote activation of murine myofibroblasts (43), control permeability of the blood brain barrier (44), facilitate migration of monocytes across the blood-brain barrier into the central nervous system (45) and coordinate macrophage migration under inflammatory conditions (46). Interestingly, this diversity in function appears to be dependent on t-PA interaction with LRP, which initiates activation of signalling cascades, whilst not always requiring the

proteolytic activity of t-PA. Expression of both t-PA and PAI-2 by monocytes, macrophages and myeloid progenitors within normal bone marrow (5), suggests a physiological role for PAI-2 as an inhibitor of t-PA associated with derivatives of the myeloid stem cells. The differential endocytosis mechanisms identified in our work suggest that while PAI-1 and PAI-2 both function similarly in the control of plasmin generation by t-PA at the cell surface, they may have opposing effects on the non-classical roles of t-PA and activation of signalling cascades via LRP or indeed VLDLR.

The molecular mechanisms and biological significance of a role for VLDLR in the endocytosis of t-PA or t-PA:PAI complexes are poorly understood. VLDLR is not found within the liver; however, it is expressed at varied levels throughout the body (3). Therefore, whilst the SPR analyses indicated that t-PA and t-PA:PAI bound to

Table 3: Comparison of t-PA/t-PA:PAI to u-PA/u-PA:PAI affinities for LRP and VLDLR.

| Analyte | LRP | VLDLR |
|------------|------------|---------------------------|
| | KD (nM) | KD (nM) |
| t-PA | 67 | 124 |
| t-PA:PAI-2 | 66 | 131 |
| t-PA:PAI-1 | 21 0.95 | 139 23 |
| u-PA | 200 * | 31.2 ^ |
| u-PA:PAI-2 | 32.6 * | 4.68 ^ |
| u-PA:PAI-1 | 0.226 € | 1.6 € 84.8 ^ 1.51 ^ |

Previously published data: * (36), ^ (37), € (47).

What is known about this topic?

- Peripheral blood monocytes and macrophages express and secrete t-PA and u-PA which results in cell surface plasmin generation.
- Plasmin activation at the cell surface of monocytes and macrophages contributes to a wide range of downstream physiological functions. Early research focused on the role of the u-PA in monocyte biology; however, it is now known that t-PA facilitates migration of myeloid cells from the bone marrow, monocytes and peritoneal macrophages in response to inflammatory mediators.
- Recent research has shown the importance of t-PA within the central nervous system, where is also known to activate microglia (macrophage of the CNS) and facilitate migration of monocytes across the blood-brain barrier.
- The SERPINS PAI-1 and PAI-2 are known to inhibit t-PA.
- The main physiological inhibitor of t-PA is PAI-1; however, PAI-2 is also known to form inhibitory complexes with this PA. Research has focused on the role of PAI-1 in controlling t-PA-facilitated plasmin generation and the physiological consequences associated with this endocytosis of this serpin:protease complex, via LRP. However, with respect to myeloid biology, previous publications in this area of research have been inconsistent and incomplete.

What does this paper add?

- This paper clarifies the role of both PAI-1 and PAI-2 in the endocytosis of t-PA from the cell surface of peripheral blood monocytes.
- Both t-PA:PAI-1 and t-PA:PAI-2 complexes are cleared from the cell surface via the low-density lipoprotein receptor family members.
- Surface plasmon resonance analysis indicates that the very-low-density-lipoprotein receptor may also facilitate endocytosis of t-PA and t-PA:PAI complexes. All previous research has focused on LRP as the endocytic receptor for t-PA or t-PA:PAI-1 complexes.
- Whilst both PAI-1 and PAI-2 are able to inhibit t-PA at the cell surface the rate of clearance of these ligands is markedly different.
- This data provides some novel mechanistic insight into differential endocytosis of t-PA, t-PA:PAI-1 t-PA:PAI-2, which suggests that while PAI-1 and PAI-2 both function similarly in the control of cellular plasmin generation by t-PA, they may have disparate effects on the functional consequences of t-PA endocytosis.

Abbreviations

t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; PAI-2, plasminogen activator inhibitor type-2; LDLR, low-density-lipoprotein receptor; LRP, low-density-lipoprotein receptor-related protein; VLDLR, very-low-density-lipoprotein receptor; PBM, peripheral blood monocytes; SPR, surface plasmon resonance; RAP, receptor associated protein.

VLDLR with approximately two-fold lower binding affinities than to LRP (Table 2), it is plausible that VLDLR may also play an important role in local clearance of t-PA and t-PA:PAI complexes. This could be through removal of proteolytic activity and/or potentiation of down stream signalling cascades through cell surface interactions with these ligands.

Overall, we have shown that the serpins PAI-1 and PAI-2 are efficient inhibitors of cell associated t-PA and that these protease:serpin complexes are indeed endocytosed, predominantly via an LDLR-dependent pathway on PBMs. Identification of potential downstream consequences associated with the differential endocytosis patterns observed in this study, may offer new insight into the role of these cells in inflammatory environments and the prevalence of myeloid diseases.

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