

Tissue Plasminogen Activator in Human Megakaryocytes and Platelets: Immunocytochemical Localization, Immunoblotting and Zymographic Analysis

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Key words

Fibrinolysis – Immunoperoxidase – Megakaryocytes – Platelets – Immunoblotting

Summary

Two approaches were used to identify and characterize the presence of tissue plasminogen activator (t-PA) in megakaryocytes and platelets. We investigated the fibrinolytic activity of human megakaryocytes (MK) and platelets. The presence of t-PA antigen in megakaryocytes and platelets was demonstrated using immunocytochemical techniques and polyclonal or monoclonal antibodies specific for t-PA. When cells were applied to fibrin plates, lysis zones developed around isolated human megakaryocytes, whereas no fibrinolytic activity appeared when either intact washed platelets or platelet lysate were deposited. After SDS-PAGE of platelet and MK extracts (Triton X-100) immunoblotting and peroxidase staining identified t-PA antigen in several bands. Zymographic analysis of SDS-PAGE carried out on fibrin film overlays identified one or two zones corresponding to free or complexed t-PA. These results indicate that t-PA is present in platelets as well as in the precursor cells, however, in platelets, t-PA may not be immediately available for plasminogen activation and fibrin degradation. From our findings and from previous work of others, it appears that platelets may either activate or inhibit the fibrinolytic system. Therefore the conditions of plasminogen activation by platelet t-PA and plasmin inhibition by platelet α_2 -antiplasmin or other inhibitors have to be precised before the role of platelets in clot dissolution is understood.

The physiological role of platelets in fibrinolysis and clot dissolution remains unclear. In 1953, the antifibrinolytic activity of blood platelets was demonstrated (1) and in the early 1960's a fibrinolytic activity, increasing with platelet concentration in the experimental system, was shown (2, 3). In 1979, it was demonstrated that metabolically active platelets were necessary for platelets to play a role in the fibrinolytic system (4). More recently it was established by Plow and Collen (5) that the specific plasmin inhibitor, α_2 -antiplasmin is a constituent of platelet α -granules.

In the present study, we investigated the fibrinolytic components and activity of human megakaryocytes and platelets, using zymographic and immunochemical techniques. We report here our observations that human megakaryocytes and platelets contain tissue plasminogen (t-PA) which possesses fibrinolytic activity.

Abbreviations:

t-PA: tissue plasminogen activator; SDS PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; MW: Molecular weight.

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Materials and Methods

Reagents

Acrylamide, bis (N,N'-Methylene-bis acrylamide), ammonium persulfate, temed, Sodium dodecylsulfate (SDS)-Page standards, Triton X-100, Tween 20 agarose (Biorad). Antigoat IgG coupled to peroxidase (Biosys, France). Plasminogen, fibrinogen (Kabi, Sweden). Percoll (Pharmacia). Thrombin (Roche, Switzerland). Aquamounts (Gurr, England). Eukitt (Kindler, Germany). Glutaraldehyde, paraformaldehyde (Taab). BA 85 membrane filters (Schleicher-Schuell, Germany). 4-Chloro-1-naphthol, 3-3' diaminobenzidine (Sigma).

Megakaryocytes were concentrated as described by Rabellino et al. (6) and modified as previously published (7). Human rib fragments were obtained after thoracic surgery, and megakaryocytes were separated on Percoll density gradients.

Platelets separated from human hypercitrated (130 mM citric acid, 126 mM trisodium citrate, 250 mM sucrose) blood, with or without Trasylol (10 U/ml) were washed twice as described previously (8).

The pellet was resuspended in 0.1% Triton X-100 in saline and centrifuged 10 min at 12,000 g to obtain the Triton-extracts.

Polyclonal Antibodies

- Goat IgG antihuman t-PA from melanoma cells, purified on affinity chromatography (Biopool, Sweden) was diluted to 60 μ g/ml.
- Rabbit antihuman t-PA antibodies, against human melanoma cells, used in immunoblotting, were a gift from J. Hauert, Lausanne (25 μ g/ml).

Monoclonal Antibodies

- Monoclonal antibody δ 12D (9) was raised against human tissue plasminogen activator (t-PA), purified from human blood after venous occlusion and is of IgG1 isotype, isolated from ascitic fluid, by ammonium sulfate precipitation.

- Monoclonal antibodies 4F9 is of IgG1b isotype and raised against von Willebrand factor (7, 8, 10). It was purified from ascitic fluid. The concentration was 11 mg/ml. Monoclonal antibodies were diluted 1:100 to 1:1000.

The complementary antibodies were coupled to peroxidase and were specific for each animal species (biosys-France). Controls were performed using purified IgG of non immunized animals and was specific for each antibody.

Immunoperoxidase Studies

Smears of platelets and megakaryocytes were fixed in cold acetone 10 min or in 2% paraformaldehyde-0.1% glutaraldehyde in phosphate buffer 20 min at room temperature and washed in 0.2 M glycine solution to quench free aldehyde groups. Sections were then overlaid with the specific antibodies and incubated overnight at 4°C. After extensive washing in Tris buffer saline (20 mM Tris-0.1 M phosphate buffer) pH 7.4, the indirect immuno-peroxidase method was used, including permeabilisation of the smears (0.1% Triton X-100 for 4 min after aldehyde fixation). Peroxidase conjugated antibodies (1:40) were applied for 1 h at 24°C. The peroxidase was detected with diaminobenzidine (0.3 mg/ml in 0.01% H₂O₂) for 3–5 min. After washings in buffer, sections were counterstained with Harris' hematoxylin.

Fibrinolysis Studies

– *Fibrin agar plates* with and without plasminogen were prepared as described by Schill and Shumacher (11).

– *Fibrinolytic activity of megakaryocytes* was studied on “fibrinolytic autographs” according to Todd (12) with the following modifications: microscopic slides were overlaid with 300 μ l of human fibrinogen solution (2 mg/ml) with and without plasminogen. Fibrinogen was then clotted with 20 μ l of thrombin (1 U/ml). Fibrin films were stored 1 h in a moist chamber at 4°C. Suspensions of megakaryocytes were deposited on the slides, incubated 30 min at 37°C and fixed in 2% paraformaldehyde for 5 min. After washings in phosphate buffer saline (PBS) followed by water, slides were counterstained with Harris’ hematoxylin and mounted in Aquamount.

Polyacrylamide gel electrophoresis was performed according to Laemmli (13) using a 10% polyacrylamide gel of 13 \times 18 \times 0.75 cm and a 4.5% stacking gel. Samples were diluted in sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue). Diluted samples (40 μ l) were applied on stacking gels; electrophoresis was run overnight at 8 mA per gel of 4 h at 20 mA. After completion of electrophoresis gels were used either for immunoblotting (gel I) or, without reducing agent for zymographic analysis (gel II).

Gel I was placed directly in contact with BA 85 membrane filters and blotted in a Biorad Trans-blot cell overnight in Tris-Glycine-methanol buffer (25 mM – 192 mM – 20% v/v) at 125 mA.

Gel II was incubated 1 h in 2.5% Triton X-100 and washed extensively in water. Fibrinolytic activity was revealed by zymographic analysis (14): the gel was applied to a fibrin plate, then incubated at 37°C in a moist chamber for 10–16 h. To stop the reaction, the proteins were fixed with 3% paraformaldehyde, washed in water and either subjected to dark field visualisation or stained with 0.1% amido-black in 70% methanol, 10% acetic acid (15).

Immunological Detection of Antigens on Nitrocellulose Blots

Immunoblots of electrophoretically separated proteins were saturated in blocking buffer: phosphate saline, bovine serum albumin, Tween 20 (0.1 M, 3%, 0.1%) for 1 h. They were then incubated with specific antibodies to t-PA (1:100) or with anti α_2 -antiplasmin (1:100). The presence of immune complexes was revealed by peroxidase conjugated anti IgG (1:200) in blocking buffer for 1 h. The peroxidase staining was

developed either with 4-Chloro-1 naphthol 0.3% in PBS or 0.4 mg/ml DAB and 0.3% H₂O₂. After washing blots were dried and stored protected from light.

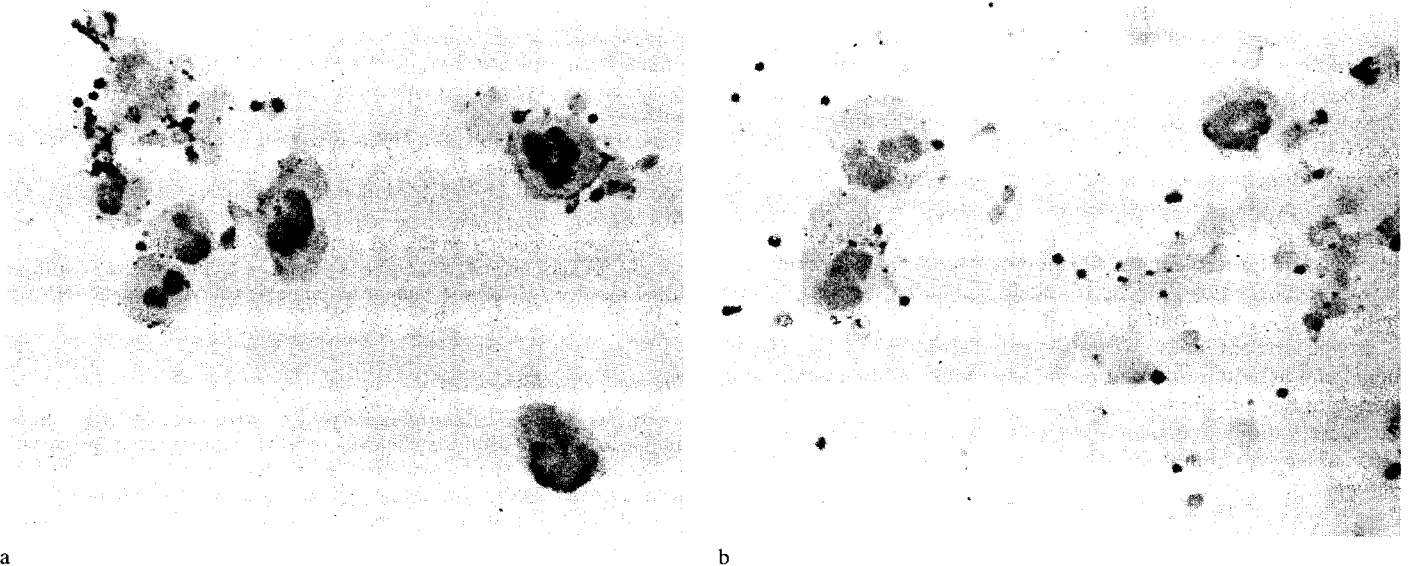
Results

Tissue plasminogen activator (t-PA) in megakaryocytes isolated from human ribs and human platelets isolated from normal blood (Fig. 1) was visualized by an immunoperoxidase reaction with polyclonal or monoclonal antibodies specific for t-PA. As shown in Fig. 1, in megakaryocytes, a diffuse cytoplasmic staining appeared either with polyclonal (Fig. 1a) or monoclonal (Fig. 1b) antibodies. This staining pattern was different in localization from that of von Willebrand factor, investigated with the same method, using a specific monoclonal antibody to von Willebrand factor (4F9) which showed a granular dense staining (Fig. 1c). Incubation with either non immunized goat IgG (Fig. 1d) or with irrelevant myeloma X63 IgG failed to show staining (Fig. 1e) of the megakaryocytes.

In platelets, the peroxidase staining after incubation with polyclonal antibodies to t-PA, confirmed the presence of t-PA (Fig. 1f) in contrast to the absence of staining in control platelets incubated with non immunized goat IgG (Fig. 1g).

Fibrinolytic activity developed by megakaryocytes on fibrin films was clearly seen as depicted in Fig. 2a as a clear zone of fibrinolysis around each isolated cell. In contrast no fibrinolysis was detected around megakaryocytes applied either on fibrin plates which did not contain plasminogen (not shown), or on fibrin plates incubated with specific polyclonal antibodies to t-PA (Fig. 2b). When platelets of platelet lysate were deposited on fibrin, no fibrinolytic areas developed even after 24 h incubation (not shown).

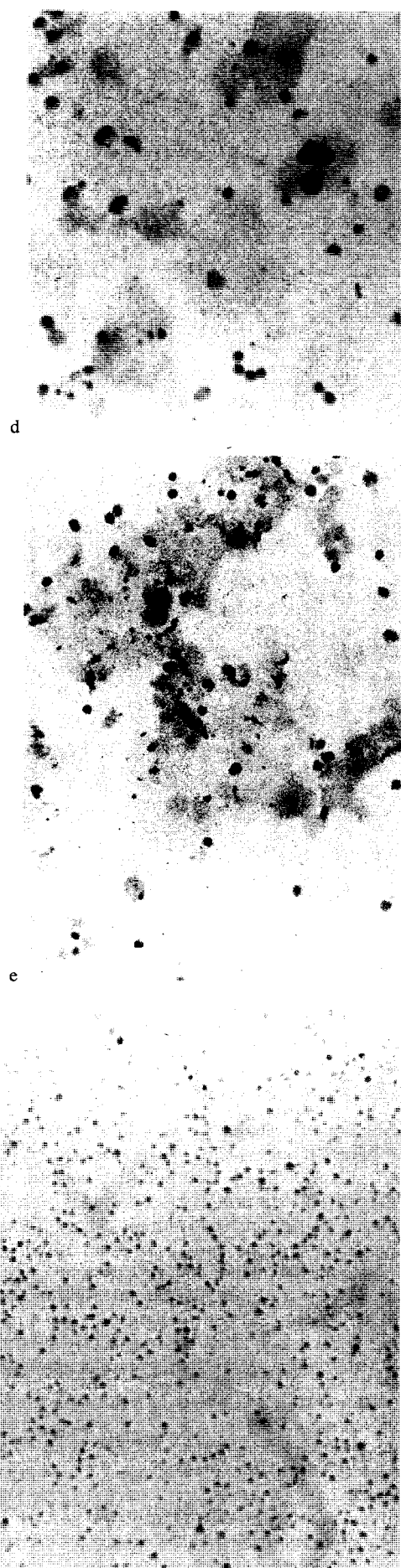
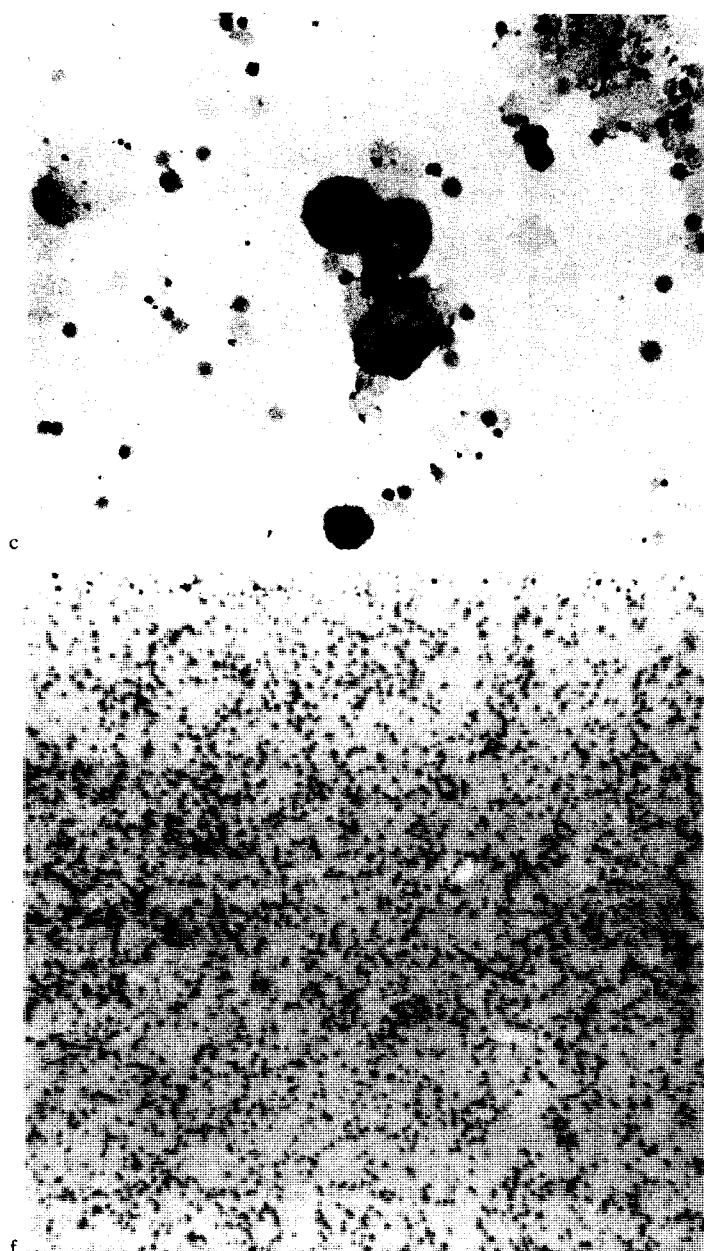
In contrast zymographic analysis of platelet extracts after Triton X-100 extraction and electrophoretic separation of proteins (SDS-PAGE) identified two bands of plasminogen activator by two lysis zones; these two bands of fibrinolysis appeared in



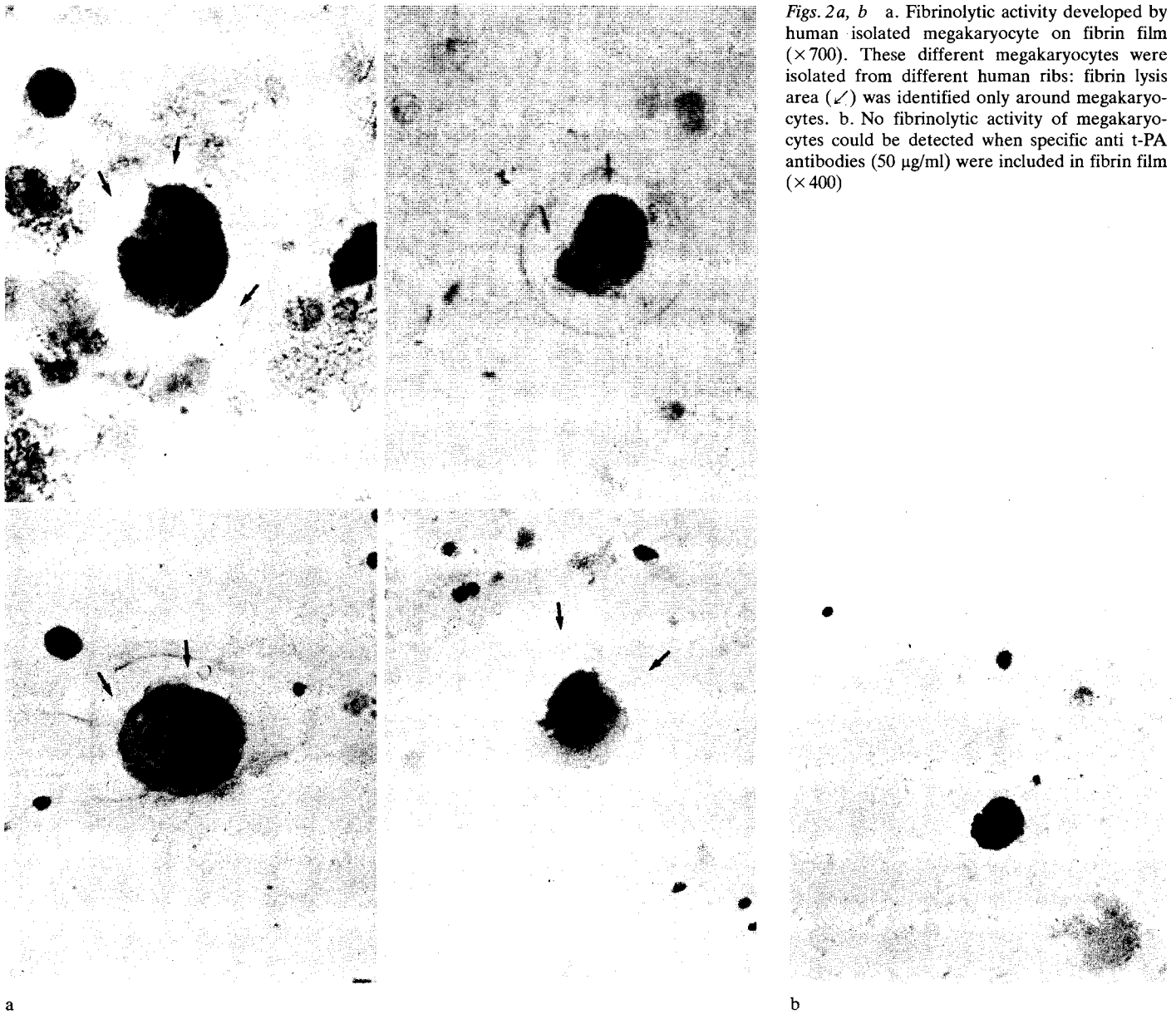
Figs. 1a–g Immunoperoxidase staining of human isolated megakaryocytes and platelets on smears after incubation with: a. megakaryocytes incubated with polyclonal anti t-PA antibodies (\times 400); b. megakaryocytes incubated with monoclonal anti t-PA antibodies (\times 400); c. megakaryocytes incubated with a monoclonal antibody to von Willebrand factor 4F9 ($6 \times$ 400); d. megakaryocytes incubated with non-immunized goat IgG (\times 400); e. megakaryocytes incubated with irrelevant myeloma X 63 IgG (\times 400); f. platelets incubated with polyclonal anti t-PA antibodies (\times 640); g. platelets incubated with non-immunized goat IgG (control) (\times 640)

black (Fig. 3). In some platelet lysates a 90 kDa lysis band with more or less intensity was detected. In Fig. 3, six different platelet lysates from six different normal subjects were allowed to migrate. Relative intensity of the 90 kDa bands showed individual variations of free and complexed t-PA for the same platelet count. All the preparations were performed in the presence of protease inhibitors. The active band of low molecular weight was found to migrate at an apparent faster position than the purified t-PA from melanoma cells (first lane). The activity observed in the lower bands corresponds to an apparent molecular weight of 55 kDa, whereas the other band was identified in each lysate in the zone of 90 kDa.

These bands of fibrinolytic activity in platelet extracts correspond to t-PA. This was confirmed by immunoblotting of SDS-PAGE on nitrocellulose and incubation with specific polyclonal rabbit anti t-PA antibodies (a gift from J. Hauert; Lausanne), which showed (Fig. 4) an identical migration to purified t-PA. It is also shown in Fig. 4 that different platelet extracts exhibit the same band which is immunologically related to t-PA after



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Figs. 2a, b a. Fibrinolytic activity developed by human isolated megakaryocyte on fibrin film ($\times 700$). These different megakaryocytes were isolated from different human ribs: fibrin lysis area (\sphericalangle) was identified only around megakaryocytes. b. No fibrinolytic activity of megakaryocytes could be detected when specific anti t-PA antibodies ($50 \mu\text{g/ml}$) were included in fibrin film ($\times 400$)

reduction. One band was visualized by peroxidase staining in the 60 kDa and one band in the 90 kDa region. Reduction with DTE of platelet extracts modified the position of platelet t-PA in comparison with non reduced samples seen on the zymogram (Fig. 3). Our observation of multiple bands for t-PA antigen are in agreement with the findings of other authors (14, 15): the lower and higher MW bands could correspond respectively to free t-PA and to t-PA complexed with an inhibitor. Polyclonal anti t-PA from Biopool (Sweden) demonstrated comparable results, however specific polyclonal anti-urokinase IgG used instead of anti t-PA did not cross react with platelet extracts (data not shown).

Triton extracts of isolated megakaryocytes (about 2×10^9 cells) was submitted to the same processing as platelet extracts and the specific polyclonal anti t-PA (from J. Hauert) revealed by Western blotting the presence of t-PA. In non reduced sample, a double band was observed, one corresponding closely to t-PA from melanoma and the other to the position of t-PA observed in platelet extracts. No band complexed t-PA with an inhibitor was observed in megakaryocyte lysate in the zone of 90 kDa.

Discussion

The combination of procedures described in this paper demonstrates that t-PA is present in human megakaryocytes and platelets. Since t-PA is present in megakaryocytes, it seems likely that platelet t-PA may not be adsorbed from plasma or after endothelial cell release but may originate directly from the precursor megakaryocytic cells, although t-PA synthesis by megakaryocytes has not yet been demonstrated. Peroxidase staining suggests a diffuse cytoplasmic localization of t-PA in megakaryocytes and platelets, contrasting with the granular localization of proteins such as von Willebrand factor.

Our studies of the fibrinolytic activity of megakaryocytes and platelets on fibrin plates have raised the problem of the availability of plasminogen activator which does not appear to be immediately or directly active in platelets or platelet lysates. Our results indicate that in megakaryocytes fibrinolytic activity is directly demonstrable, but in platelets, inhibitors of t-PA may be present. These inhibitors may be found at two different steps: specific t-PA inhibitor or fast acting inhibitor (18), but also

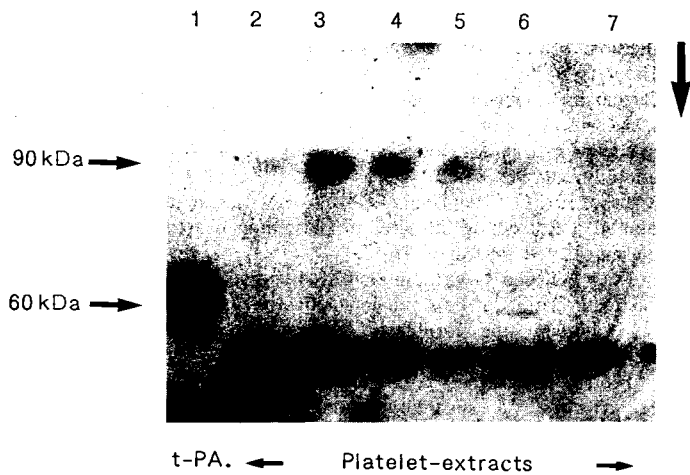
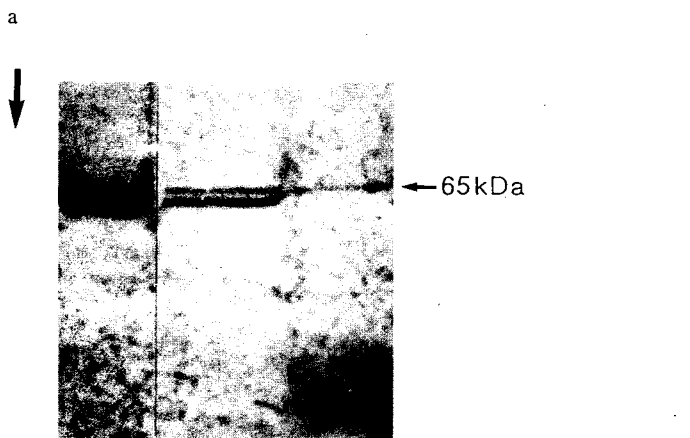
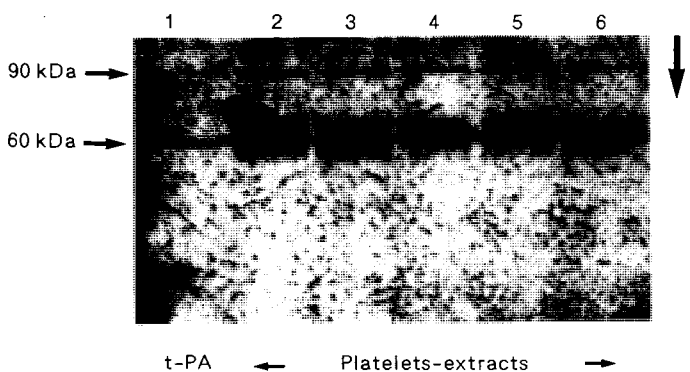


Fig. 3 Zymographic analysis of t-PA in platelet Triton extracts after SDS-PAGE: different platelet extracts from 6 normal individuals (lanes 2 to 7) in comparison with purified t-PA from melanoma cells (lane 1). Lysis zones appear in black on the film



Figs. 4a, b Western blots after SDS PAGE of cell extracts incubated with specific polyclonal antibodies anti t-PA (a gift from J. Hauert, Lausanne). a. Immunoblotting of platelet Triton extracts. After reduction with DTE, peroxidase staining visualized the t-PA antigen present in platelets, one band in the 60 kDa region, which represents the MW of free t-PA (line 1) and one band in the 90 kDa region which is related to complexed t-PA (lines 2 to 6). b. Immunoblotting of megakaryocyte Triton extract (line 2) without DTE in comparison with platelet extracts without DTE (line 1) and purified t-PA (line 3) from melanoma cells: the reaction is immunologically related to free t-PA in megakaryocytes

plasmin inhibitors, mainly α -2 antiplasmin as it has been demonstrated by Plow and Collen (5), and also α -2 macroglobulin and α -1 antitrypsin (19). However, in platelets, inhibitors can be dissociated by SDS-PAGE and functional t-PA recovered as it is demonstrated on zymographic analysis: distinct zones of lysis were developed in the 60 kDa region which represent the MW of t-PA and in the 90 kDa region which represents complexed t-PA. The relative amount of free and complex t-PA varies from one platelet extract to another as it was shown in Fig. 3. This may be due to variation of platelet t-PA and t-PA inhibitor concentration from one individual to another or variable dissociation during SDS-PAGE and zymography. On immunoblot, several bands could be identified by specific polyclonal antibodies to t-PA suggesting that t-PA migrates in association with other proteins. This could be an artifact due to specific aggregation of anti t-PA to proteins or it could also be, as suggested by Kruithof et al. (16), SDS electrophoresis dissociated complexed t-PA. Nevertheless the development of lysis zones is correlated to two precise localizations: free t-PA and complexed t-PA. In platelet lysate, free t-PA was found to have a lower MW than purified t-PA from melanoma cells or t-PA present in human plasma. This is in agreement with the finding of Granelli-Piperno and Reich (15) who described in animal species a lower MW of t-PA in cultured cells than t-PA in plasma of the same species. It was suggested by these authors that a moderate enzymatic degradation could occur in cell cultures. However, in our hands cell lysate with and without enzyme inhibitors showed no difference in the MW of free t-PA. All these results raised several questions. The first concerns the apparent modification of t-PA activity from megakaryocytes to platelets. In megakaryocytes t-PA is functionally available for plasminogen activation, whereas in platelets it is not. In platelets, no free plasmin is available for fibrin degradation. It is possible that during the differentiation of platelets from megakaryocytes, t-PA is complexed to an inhibitor, as suggested by the immunoblotting analysis. A second important question concerns the mechanisms by which platelet t-PA is released from the cell to function in fibrinolysis. The process of platelet t-PA availability is probably different from the mechanism by which other platelet proteins are released such as β -thromboglobulin or platelet derived growth factor stored in alpha granules. Platelet t-PA is scattered in the cytoplasm and can be made available after cell disruption. A third but main question concerns the conditions for t-PA dissociation from the inhibitor to which it appears to be complexed. These questions need to be addressed to improve understanding of the role played by platelets in clot or thrombus dissolution.

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