Plasminogen Activator Inhibitor–1 mRNA Is Expressed in Platelets and Megakaryocytes and the Megakaryoblastic Cell Line CHRF-288

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Plasminogen activator inhibitor–1 (PAI-1), the primary physiological inhibitor of tissue plasminogen activator (tPA) and urokinase, is synthesized by a variety of cells, including endothelial cells, smooth muscle cells, cultured hepatocytes, granulosa cells, and a number of malignant cell lines. PAI-1 is also present in the platelet α-granule and is released on platelet activation. The source of platelet PAI-1 is unknown. Platelet α-granule proteins may either be synthesized by megakaryocytes or taken up from the plasma. β-Thromboglobulin, platelet factor 4, and von Willebrand factor (vWF) are examples of proteins synthesized by megakaryocytes, while immunoglobulin G (IgG), albumin, and fibronectin are examples of proteins acquired by endocytosis.

PAI-1 protein has been identified in megakaryocytes. Indirectly, Jeanneau and Sultan found tPA in complexed form, presumably to PAI-1, by zymographic and Western blot analysis of megakaryocyte cell extracts. More recently, Simpson et al reported positive PAI-1 immunostaining of human platelets and megakaryocytes. However, Perez et al did not find PAI-1 mRNA on Northern blot analysis of human platelet RNA, nor could they amplify PAI-1-specific cDNA from reverse-transcribed (RT) human platelet RNA, leading them to question whether PAI-1 is synthesized by megakaryocytes. We now report studies confirming the synthesis of PAI-1 protein by human megakaryocytes and show that human platelets and guinea pig megakaryocytes contain PAI-1 mRNA. We also report that PAI-1 mRNA expression is induced in the megakaryoblastic cell line CHRF-288 by phorbol ester treatment.

**Methods**

**Megakaryocyte Isolation**

Guinea pig megakaryocytes were isolated as previously described. Briefly, bone marrow was obtained from the long bones of animals weighing 300–500 g and separated by density gradient centrifugation and two successive velocity sedimentations on bovine albumin gradients. Megakaryocytes were counted, and contamination was assessed by phase-contrast microscopy. Viability was evaluated by trypan blue exclusion. The isolated cells contained 85% megakaryocytes by cell number and >98% megakaryocytes by cell volume.

Human megakaryocytes were propagated from peripheral blood in liquid culture as previously described. Cultures were initiated in E. Mazur's laboratory, sent to P. Schick's laboratory on day 11, and then incubated for 24 hours before separation by the Celsep procedure. The Celsep procedure, described in detail elsewhere, allows the isolation of subpopulations of megakaryocytes according to size. The largest cells are...
isolated in fractions 2–6, and there is a progressive decrease in size in subsequent fractions. Since megakaryocytes are larger than other bone marrow cells, they are recovered in fractions 2–18 and non-megakaryocytic cells are isolated in fractions 19–22. Platelets are not present in any fraction. In the culture before separation by the Celsep procedure, 9.6% of the cells were megakaryocytes. After purification, fractions 2–18 were combined and used for Western blot analysis. Together these fractions contained ≥55% megakaryocytes. Megakaryocytes were identified by morphology and vWF immunostaining. Trypan blue exclusion revealed that the megakaryocytes were 79% viable.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated and propagated as previously described. Total cellular RNA was extracted at the third passage. Human erythroleukemia (HEL) cells were obtained from the American Type Culture Collection (ATCC TIB 180, HEL 92.1.7) and propagated in RPMI 1640 (GIBCO BRL, Gaithersburg, Md.) with 10% fetal bovine serum (Hyclone Labs, Logan, Utah). CHRF-288 cells, kindly provided by M. Lieberman, Children's Hospital Medical Center, Cincinnati, Ohio, were propagated in Fischer's medium (GIBCO BRL) with 20% horse serum (GIBCO BRL). All cultures were maintained in a 5% CO₂ humidified atmosphere at 37°C.

**Western Blot Analysis**

Megakaryocytes were solubilized in 0.125 M tris(hydroxymethyl)amino methane hydrochloride (Tris HCl), pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% β-mercaptoethanol. Approximately 42 μg protein, representing ~42,000 megakaryocytes, was subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of SDS on a 5% gel. Proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Richmond, Calif.); incubated with a rabbit polyclonal anti-human PAI-1 antibody kindly provided by D. Ginsburg, University of Michigan, Ann Arbor; and developed using a horseradish peroxidase-conjugated IgG produced no band. Controls using rabbit IgG produced no band.

**Immunoprecipitation**

Isolated megakaryocytes were incubated with 100 μCi/mL of Expre[35S]S Protein Labeling Mix ([^35S]methionine; DuPont NEN, Boston, Mass.) for 17 hours at 37°C. After incubation, cells were disrupted by SDS (0.2%) in the presence of protease inhibitors (4 mM EDTA, 2 mM iodoacetic acid, 2 mM N-ethylmaleimide, and 4 mM phenylmethylsulfonyl fluoride). Gelatin Sepharose (Sigma Chemical Co., St. Louis, Mo.) was added to the cell lysates for 1.5 hours at room temperature and then removed by centrifugation. The samples were then incubated for 1.5 hours at room temperature with protein A-Sepharose beads (Sigma) that had been precubicated for 30 minutes with the polyclonal anti-PAI-1 antibody used for the Western blot analysis. The beads were washed, placed in treatment buffer (0.625 M Tris HCl, pH 6.8, 10% glycerol, and 2% SDS), boiled for 3 minutes, and separated by 10% SDS-PAGE. Protein synthesis was detected by fluorography.

**Platelet RNA Isolation and Polymerase Chain Reaction**

Platelet-rich plasma was prepared from 40 mL of acid/citrate/dextrose (ACD)–anticoagulated peripheral blood by centrifugation at 100g for 10 minutes. Washed platelets were prepared by centrifugation at 2,500g for 10 minutes to form a platelet pellet. The pellet was washed twice with ACD, and total RNA was prepared. Alternatively, the platelet-rich plasma was applied to a Sepharose 2B (Pharmacia LKB Technology, Piscataway, N.J.) column that had been equilibrated with calcium- and magnesium-free Tyrode's buffer (0.137 M NaCl, 3 mM KCl, 0.4 mM NaH₂PO₄, 12 mM NaHCO₃, 14.7 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) containing 0.1% bovine serum albumin, to which ethylene glycol-bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid and EDTA (0.5 mM final concentration of each) had been added. The platelet-containing fractions were collected and centrifuged at 2,500g for 10 minutes, and RNA was prepared from the platelet pellet by solubilization in guanidine HCl as previously described.

The RNA was RT amplified by the polymerase chain reaction (PCR) as previously described. Briefly, five units of the isolated RNA was used as a template with 10 units of avian myeloblastosis virus reverse transcriptase (Seikagaku America, Rockville, Md.) for 1 hour at 41°C in 50 mM Tris HCl, pH 8.3, 50 mM KCl, 8 mM MgCl₂, 10 mM dithiothreitol, and deoxynucleotide triphosphates at 500 μM each in a total volume of 50 μL with 0.06 A₉₀₀ unit (number of units at 260-nm absorbance) of oligodeoxynucleotide 16 as the primer. Ten microliters of the reverse-transcriptase reaction mixture was adjusted to PCR buffer conditions in a total volume of 50 μL with 0.02 A₁₀₀₀ unit each of PCR primer and 5 units of Taq polymerase (Thermus aquaticus DNA polymerase, Perkin-Elmer Cetus, Norwalk, Conn.). PCR was performed for 30 cycles in an automated thermocycler (Perkin-Elmer Cetus) with cycle times of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C. Primers used were prepared as based on published nucleotide sequences and included the following: PAI-1 (335–364, 667–638 [2]), vWF (7,116–7,155, 7,824–7,785 [22]), glycoprotein Iba (331–360, 558–592 [22]), and tPA (1,040–1,080, 1,543–1,503 [24]). All primers except those for glycoprotein Iba span an intron to control for amplification of residual DNA.

**RNA Isolation and Evaluation**

Total cellular RNA was prepared by immediate solubilization of the cell fractions in guanidine HCl (International Biotechnologies, Inc., New Haven, Conn.) as previously described. Except where noted, 10 μg of each sample was electrophoresed under denaturing conditions in a formaldehyde-containing gel and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, Ill.) by Northern blotting. The RNA was fixed to the membrane with UV irradiation. The blots were prehybridized in 1 M NaCl, 0.1% SDS, 1.5 mg/mL herring sperm DNA, and 10% dextran for 3 hours at 65°C and then hybridized with the appropriate radiolabeled probe at 65°C for 12–24 hours in the prehybridization solution with the addition of 1.5 mg/mL sonicated herring sperm DNA. Blots were washed to various stringencies, depending on the probe used, in a solution containing 0.1–0.2×
saline-sodium citrate with 0.1% SDS, 1 mM EDTA (pH 8), and 10 mM sodium phosphate (pH 6.8) at 55°-65°C and analyzed by autoradiography. Densitometric analyses of the autoradiographs were performed using a Hoefer GS300 scanning densitometer and the GS365W data system (Hoefer Scientific Instruments, San Francisco). Densitometry results for PAI-1 mRNA signals were normalized to scans of the same blot reprobed with the cDNA for phosphoglycerate kinase to control for RNA quantification.

Guinea pig PAI-1 and vWF cDNAs and human PAI-1 and phosphoglycerate kinase cDNAs were used as probes. Guinea pig-specific PAI-1 and vWF probes were prepared after human–guinea pig cross-species hybridization did not produce a signal sufficient for evaluation. The guinea pig PAI-1 probe was prepared using PCR to amplify PAI-1 cDNA from RT guinea pig bone marrow or lung RNA by using primers based on the human sequence (published nucleotide sequence No. 335–364 and 667–638) \[1\]). The guinea pig vWF probe was amplified from the same RT RNA by using primers based on the human sequence (nucleotides 7,116–7,155 and 7,824–7,785) \[2\]). These procedures were performed as previously described, except that relaxed annealing conditions were used for the first five cycles of the PCR (1 minute at 94°C, 1 minute at 37°C, a gradual increase over 2 minutes to 72°, and 2 minutes at 72°C), and then standard conditions as above were used for the next 25 cycles. The amplified cDNAs were labeled with \[\text{[32P]deoxycytidine triphosphate}\] using random-hexamer priming. The identity of the guinea pig PAI-1 and vWF probes was confirmed by their ability to detect the appropriately sized message in guinea pig RNA, by cross-hybridization to the appropriate message species in HUVEC RNA under low-stringency hybridization and washing, and by direct sequencing of the amplified bands using a modification of the dideoxy chain-termination method to sequence double-stranded DNA.24 The sequence of the amplified guinea pig PAI-1 fragment was approximately 90% homologous to the human sequence.

**Results**

Western blot analysis of protein from isolated human megakaryocytes with an anti-human polyclonal PAI-1 antibody revealed an immunoreactive band of approximately 45 kD, as shown in Figure 1A. The cellular extract also contained vWF immunoreactivity but was nonreactive with control IgG (data not shown). Although the megakaryocyte fraction was only 55% pure, the remainder of the cells were almost entirely small lymphocytes, and no platelets were present. Peripheral blood lymphocytes do not express PAI-1; thus, it is likely that the PAI-1 immunoreactivity is from megakaryocyte protein, thereby confirming previously published studies.

To determine whether megakaryocytes synthesize PAI-1, guinea pig megakaryocytes were incubated with \[\text{[35S]methionine/35S]cysteine}\] and immunoprecipitated with a polyclonal anti-PAI-1 antibody as shown in Figure 1B. One band of approximately 45 kD was precipitated.

We evaluated human platelets for the presence of PAI-1 mRNA. Vestigial amounts of mRNA remain in platelets when they are separated from the megakaryocyte, and analysis of this RNA has been used by a number of investigators to study megakaryocyte-derived mRNAs.25,26,27,28 PCR analysis of RT RNA prepared from washed platelets and from HUVECs revealed the presence of PAI-1 cDNA as shown in Figure 2. We were unable to amplify a tPA-specific cDNA fragment from RT platelet RNA under the same conditions but were able to amplify it from RT HUVEC RNA. Segments of vWF and glycoprotein Ibα cDNA, which are synthesized by platelets, were used without added cDNA. One tenth of the PCR reaction, fractionated on a 3% agarose gel, is shown. Size markers are shown on the left in kilodaltons.

**Figure 1.** Panel A: Western blot analysis of human megakaryocyte plasminogen activator inhibitor–1 (PAI-1). Reduced samples of sodium dodecyl sulfate (SDS)–solubilized megakaryocytes were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with a polyclonal anti–PAI-1 antibody, and developed with peroxidase-labeled goat anti-rabbit immunoglobulin G. Panel B: Immunoprecipitation of guinea pig megakaryocyte PAI-1. Megakaryocytes were incubated with \[\text{[35S]methionine/35S]cysteine}\] followed by immunoprecipitation using a polyclonal anti–PAI-1 antibody. Nonreduced samples were electrophoresed in a 10% polyacrylamide gel in the presence of SDS and visualized by autoradiography. The numbers to the left of the gels represent the molecular mass of standard proteins in kilodaltons.

**Figure 2.** Polymerase chain reaction (PCR) analysis of platelet and human umbilical vein endothelial cell (HUVEC) RNA. PCR was performed using oligodeoxynucleotide-primed, reverse-transcribed platelet and HUVEC RNA and cDNA-specific primers to amplify plasminogen activator inhibitor–1 (PAI-1), von Willebrand factor (vWF), glycoprotein Ibα (GpIba), and tissue plasminogen activator (tPA). For the control lane, reagents and the PAI-1-specific primers were used without added cDNA. One tenth of the PCR reaction, fractionated on a 3% agarose gel, is shown. Size markers are shown on the left in base pairs.
in both megakaryocytes and HUVECs, were amplified from both sources. To eliminate possible white blood cell contamination of the platelet pellet, we also gel-purified the platelets (see "Methods"). Using the PAI-1 specific primers, one appropriately sized band was amplified from this RT RNA. Sequence analysis of this product confirmed its identity as PAI-1.

To document the presence of PAI-1 mRNA in megakaryocytes, we evaluated guinea pig megakaryocyte RNA by Northern blot analysis as shown in Figure 3. Guinea pig megakaryocytes were used because of the number of cells required as well as the ability to obtain purer fractions. The megakaryocytes used were >85% pure by cell number and >98% pure by RNA or protein content, since megakaryocytes are much larger cells and contain greater amounts of RNA and protein per cell. Using a guinea pig-specific PAI-1 probe (see "Methods"), we identified one >3-kb mRNA species. Nonprimate mammals express only one PAI-1 mRNA species in contrast to the two alternatively processed mRNA species seen in primates. The blot reprobed with radiolabeled guinea pig-specific vWF cDNA revealed one >9-kb mRNA species (Figure 3). In other experiments, Northern blot analysis of RNA prepared from the isolated bone marrow cells in Celsep fractions 20–24, used as controls because they do not contain identifiable megakaryocytes or platelets, showed no mRNA species for either PAI-1 or vWF (data not shown).

Because of the difficulty in isolating and propagating megakaryocytes, investigators have developed and studied cell lines with megakaryocyte properties. HEL cells, a cell line derived from a patient with erythroleukemia, express numerous megakaryocyte proteins in addition to erythroid proteins, especially when stimulated with dimethyl sulfoxide or phorbol esters. We could not detect PAI-1 mRNA by Northern blot analysis of total cellular RNA extracted from HEL cells that were either untreated or treated for 4 days with dimethyl sulfoxide (1.25%, vol/vol) or 12-myristate 13-acetate (PMA, 160 nM) for 4 days. PMA-treated cells that became adherent (adh.) were isolated separately from cells that remained in suspension (susp.). Total cellular RNA was isolated and approximately 10 µg evaluated by Northern blot analysis with radiolabeled human PAI-1 cDNA as a probe. For the upper panel, the blot was stripped of radiolabeled PAI-1 and reprobed with phosphoglycerate kinase (PGK) cDNA as a control for RNA quantification.

Discussion

In this article we show that megakaryocytes synthesize PAI-1 mRNA, thus providing a source of platelet PAI-1. PAI-1 is present in the platelet α-granule at a 300–3,000-fold higher concentration than that found in plasma. In a review of platelet α-granule proteins, George postulated that the mechanism of α-granule acquisition is suggested by the relative concentration of the proteins in platelets and plasma. This analysis also suggests that PAI-1 would be synthesized by the megakaryocyte. We cannot, however, rule out that platelet PAI-1 is also acquired by endocytosis of the plasma protein.

Why we were able to detect PAI-1 mRNA in human platelets and Perez et al were not is unclear. This was perhaps due to increased sensitivity of our assays or to the decay of PAI-1 mRNA in the platelet samples they used. Faint PAI-1 immunoreactivity at levels much less than those found in platelets has been reported in granulocytes. Although we confirmed our platelet RNA results by using gel-filtered platelets, we cannot absolutely rule out the possibility of other cell contamination in the platelet RT PCR. However, since megakaryocytes contain PAI-1 mRNA, it is likely that platelets do also. Fay et al also used RT RNA from washed...
Platelet PAI-1 activity with platelet activation. 40 Active platelet PAI-1 activity in vivo. Vitronectin, which binds and stabilizes PAI-1-vitronectin complexes have been documented. 39 Negatively charged phospholipids are capable of activating PAI-1 and could be a mechanism for increased platelet PAI-1 activity with platelet activation. 40 Active PAI-1 could then bind the vitronectin on the platelet surface, thereby increasing the prothrombotic nature of the platelet aggregate.

tPA protein has been identified in both megakaryocytes and platelets, and tPA activity has been observed in megakaryocytes. 9,41 Cultured human megakaryocytes developed a clear zone of fibrinolysis when placed on fibrin films, and this was completely inhibited by antibodies to tPA. 39 Unlike PAI-1, which is present in the platelet α-granule, tPA is distributed diffusely in the platelet and is not released on platelet activation. 9 No studies have reported PAI-1-tPA complexes within platelets, but one study reported complexes in protein extracted from megakaryocytes. 8 Whether this occurred in vivo or after disruption of the megakaryocytes is not known. We could not detect tPA mRNA in guinea pig megakaryocyte RNA by Northern blot analysis (data not shown) or in RT platelet RNA by PCR. Either tPA is not synthesized by megakaryocytes, or tPA mRNA is present at levels not detected by these methods.

Phorbol ester treatment of HEL and CHRF-288 cells induces a more megakaryocyte-like cell with hyperplasia and increased megakaryocyte-specific protein expression. 20,21,42 Because phorbol 12-myristate 13-acetate treatment increases PAI-1 gene transcription in human hepatoma and endothelial cells, 33,44 a direct effect of phorbol 12-myristate 13-acetate on the PAI-1 gene is a possible reason for PAI-1 induction in the CHRF-288 cells. However, unlike in CHRF-288 cells in which induction occurred after 4 days in culture, in hepatoma and endothelial cells the phorbol 12-myristate 13-acetate–induced increase in PAI-1 is seen early (within 4 hours) and is transient, with PAI-1 mRNA levels returning to baseline levels by 12 hours after treatment. Since the induction of PAI-1 mRNA in CHRF-288 cells correlates in time with the cells differentiating toward more megakaryocyte-like cells, it is likely that the latter factor is important in PAI-1 induction.

Why phorbol 12-myristate 13-acetate treatment induces PAI-1 expression in CHRF-288 cells and not in HEL cells is unknown. Neither HEL nor CHRF-288 cells are pure megakaryocyte cell lines. HEL cells are triphenotypic, expressing erythroid, macrophage, and megakaryocytic markers. 42 CHRF-288 cells were derived from a solid tumor in an infant with megaloblastic leukemia and retain some characteristics of the original tumor by producing basic fibroblast growth factor and transforming growth factor–β. 29 Inducible PAI-1 expression in CHRF cells could be a characteristic remaining from the solid tumor. Alternatively, if PAI-1 is expressed late in megakaryocyte development, then its inducibility in CHRF-288 cells may reflect a more mature megakaryocytic phenotype in these cells. Phorbol ester treatment of HEL cells for longer than 96 hours or under different conditions might also result in PAI-1 induction.

As we have discussed, platelet PAI-1 is most likely derived from its parent megakaryocyte. A number of factors, including tumor necrosis factor, transforming growth factor–β, interleukin-1, endotoxin, glucocorticoids, epidermal growth factor, and acidic fibroblast growth factor, have been shown to modulate PAI-1 expression in nonmegakaryocytes. 18,20,45-49 If megakaryocyte PAI-1 expression is also modulated, then exposure of the megakaryocyte to these factors in vivo could alter the fibrinolytic potential of the subsequently produced platelet.

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References


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