The Specific Activity of Plasminogen Activator Inhibitor-1 in Disseminated Intravascular Coagulation With Acute Promyelocytic Leukemia

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In disseminated intravascular coagulation (DIC) with acute promyelocytic leukemia (APL) in the absence of severe infection, marked fibrinolysis was noted in comparison with normal levels of antithrombin III, which is a major inhibitor of the coagulation system. Increased plasminogen activator inhibitor-1 (PAI-1) antigen levels in plasma from patients with septicemia decreased the ratio of the plasma clot lysis rate induced by an anti-\(\alpha_2\)-plasmin inhibitor monoclonal antibody to the tissue-type plasminogen activator (t-PA) concentration. This decrease was not as prominent in plasma from patients with DIC, especially those with APL. To explore the character of PAI-1 in these plasmas, we measured the specific activity of PAI-1 by determining the ratio of active PAI-1 antigen to t-PA-unbound PAI-1 antigen. To calculate the amount of active PAI-1 antigen, the amount of t-PA/PAI-1 complex before and after the addition of a fixed amount of t-PA to the sample was measured by a sandwich solid-phase enzyme-linked immunosorbent assay using anti-PAI-1 and anti-t-PA monoclonal antibodies. The assay to measure total PAI-1 antigen used three monoclonal anti–PAI-1 antibodies and had similar sensitivities to free active, latent, vitronectin-bound and t-PA-bound PAI-1. The specific activity of PAI-1 decreased in patients with DIC (43.7% ± 30.6%) and in DIC cases with APL (10.3% ± 6.0%) in comparison to patients with septicemia (83.7% ± 20.2%) or normal controls (85.8% ± 27.3%). In DIC associated with APL, degraded forms of PAI-1 were detected in plasma by immunoblotting. These results suggest that a decrease in the specific activity of PAI-1 and an increase in secondary fibrinolysis result in a hyperfibrinolytic state in DIC patients with APL.

**Hemorrhage** is the second most common fatal complication in patients with acute promyelocytic leukemia (APL). The bleeding diathesis is due mainly to disseminated intravascular coagulation (DIC) initiated by the release of procoagulant activity from abnormal promyelocytes, although primary fibrinolysis mediated by elastase-like proteases may also play a part. Although thrombin generation in DIC with APL was reported, a beneficial effect of heparin for treatment still remains controversial. Many patients with APL have low leukocyte counts because of therapy against the abnormal promyelocytes, which results in severe infection in these patients, and virtually any gram-negative organism can produce severe DIC.

Therefore, we selected DIC patients with APL who did not have severe infection and examined the parameters relating coagulation and fibrinolysis. Because the fibrinogen degradation products (FDP) in the plasmas were remarkably high relative to the decrease in protein C or antithrombin III (as described later), we analyzed this abnormal hyperfibrinolysis.

Initiation of fibrinolysis is mediated by plasminogen activators and regulated by plasminogen activator inhibitor-1 (PAI-1). PAI-1 inhibits both tissue-type plasminogen activator (t-PA) and urokinase by forming a 1:1 stoichiometric complex that resists dissociation by sodium dodecyl sulfate (SDS). PAI-1 is a relatively unstable molecule and rapidly decays into a latent form that can be converted into an active molecule after treatment with denaturants. In contrast, PAI-1 in plasma seems to exist primarily in a functionally active form, and thus a good correlation has been found between PAI-1 activity and antigen in plasma. In addition, plasma vitronectin (VN) appears to bind to the coagulation system. Increased plasminogen activator inhibitor-1 (PAI-1) antigen levels in plasma from patients with severe infection, marked fibrinolysis was noted in comparison with normal levels of antithrombin III, which is a major inhibitor of the coagulation system. Increased plasminogen activator inhibitor-1 (PAI-1) antigen levels in plasma from patients with septicemia decreased the ratio of the plasma clot lysis rate induced by an anti-\(\alpha_2\)-plasmin inhibitor monoclonal antibody to the tissue-type plasminogen activator (t-PA) concentration. This decrease was not as prominent in plasma from patients with DIC, especially those with APL. To explore the character of PAI-1 in these plasmas, we measured the specific activity of PAI-1 by determining the ratio of active PAI-1 antigen to t-PA-unbound PAI-1 antigen. To calculate the amount of active PAI-1 antigen, the amount of t-PA/PAI-1 complex before and after the addition of a fixed amount of t-PA to the sample was measured by a sandwich solid-phase enzyme-linked immunosorbent assay using anti-PAI-1 and anti-t-PA monoclonal antibodies. The assay to measure total PAI-1 antigen used three monoclonal anti–PAI-1 antibodies and had similar sensitivities to free active, latent, vitronectin-bound and t-PA-bound PAI-1. The specific activity of PAI-1 decreased in patients with DIC (43.7% ± 30.6%) and in DIC cases with APL (10.3% ± 6.0%) in comparison to patients with septicemia (83.7% ± 20.2%) or normal controls (85.8% ± 27.3%). In DIC associated with APL, degraded forms of PAI-1 were detected in plasma by immunoblotting. These results suggest that a decrease in the specific activity of PAI-1 and an increase in secondary fibrinolysis result in a hyperfibrinolytic state in DIC patients with APL.

**MATERIALS AND METHODS**

**Reagents.** The following materials were purchased: Freunds' adjuvant, polyethylene glycol 1540, dimethyl sulfoxide, 2,6,10,14-tetramethylpentadecane (Prisnine), and Tween 20 (Wako Chemical, Osaka, Japan); penicillin and streptomycin (GIBCO, Grand Island, NY); calf serum, Dulbecco's modified Eagle's medium (DMEM), and 96-well polystyrene microtiter plates (Titer-tek) (Flow Laboratories, North Ryde, Australia); fetal calf serum (FCS, Filtrin, Altona, Australia); tissue culture flasks (Corning Glass Works, Corning, NY); gentamycin, bovine serum albumin (BSA) essentially fatty acid-free, globulin-free, theophylline, and protaglandin E (Sigma, St Louis, MO); solid-phase lactoperoxidase-glucose oxidase (Enzymobeads) and horseradish peroxidase-conjugated goat antimouse Ig (BioRad, Richmond, CA); CNBr-

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activated Sepharose 4B, protein A-Sepharose CL-4B, and Sepharose PD10 (Pharmacia-LKB Biotechnology, Uppsala, Sweden); collagen (Niko Bioscience, Tokyo, Japan); and 2,2′-asino-di-di-[3-ethyl-bentazolium sulfonate] (ABTS) (Kirekegaard & Perry Laboratories, Gaithersburg, MD). The mouse myeloma cell line P3-X63-Ag8-U1 (P3U1) was provided by Dr T. Watanabe (Kyushu University, Fukuoka, Japan). Aprotinin and single-chain urokinase-type plasminogen activator (scu-PA) were gifts from Mochida Pharmaceutical (Tokyo, Japan).

t-PA was purified from medium conditioned by human melanoma cells with aprotinin as described previously and had a specific activity of 550,000 U/mg protein according to the t-PA International Standard (83/517). It migrated as a single band in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions. The t-PA International Standard was supplied by the National Institute for Biological Standards and Controls (London, England). Glu-plasminogen was purified from fresh frozen plasma in the presence of 100 kallikrein inactivating units (KIU)/mL aprotinin as described. Fibrinogen was prepared according to the method of Blomback and Blomback. Vitronectin was initially prepared from fresh frozen plasma according to the method of Dahlback and Fodack. Immunopurified VN was also prepared from fresh frozen plasma by ammonium sulfate precipitation (50% saturation) and affinity chromatography on a column containing anti-VN polyclonal antibody immobilized on Sepharose. Human PAI-1 was purified from the conditioned media of cultured human umbilical vein endothelial cells as previously described. Except for immunopurified VN, the purified proteins appeared to be greater than 98% homogeneous on SDS-PAGE. Immunopurified VN had a significant amount of material that smeared from the top of the gel to the 65,000-dalton polypeptide on SDS-PAGE. Protein concentrations were determined from their respective extinction coefficients at 280 nm. Protein concentrations of t-PA and PAI-1 were determined by amino acid analysis by the phenylthiocarbamyl method as described using the PICO.TAG system (Waters Associates, Milford, MA) after hydrolysis for 24 hours in HCl vapor at 110°C. The quantities of t-PA and PAI-1 were calculated based on their amino acid composition as deduced from their cDNA sequences and assumed molecular weights of 70,000 and 50,000, respectively.

Monospecific antisera against t-PA, scu-PA, PAI-1, and VN were prepared in rabbits as described previously. The t-PA and PAI-1 complex was purified as follows: Confluent cultures of human endothelial cells (1.5 × 10^6/cm^2) were washed three times with serum-free medium 199 and then incubated in serum-free medium containing 1 μg/mL t-PA for 6 hours. This conditioned medium was applied to an affinity column containing anti-t-PA antibody immobilized on Sepharose. Bound antigen was eluted with 3 mol/L KSCN and was further purified using anti-PAI-1 antibody-immobilized Sepharose as described. The quantity of PAI-1 present as a complex was calculated from the amount of t-PA present, assuming a 1:1 stoichiometric complex. The amount of t-PA was measured by ELISA using anti-t-PA monoclonal antibodies (MoAbs) JTA-1 and JTA-2 as described. In this ELISA, the amount of t-PA complexed with PAI-1 can be measured by using the same standard curve constructed for free t-PA as described. The level of t-PA was also confirmed by the commercial ELISA kit Imulysel t-PA (Biopool AB, Umea, Sweden).

MoAbs against PAI-1, using either t-PA/PAI-1 complex or latent PAI-1 as an antigen, were raised by standard methods using BALB/c mice and the mouse myeloma cell line P3U1 as described. The dissociation constants (kd) of 125I-labeled MoAbs for the latent PAI-1 and t-PA/PAI-1 complex were assessed by a solid-phase assay as described, according to the Frankel and Gerhard method. The competition between MoAbs (JTAI-1, JTAI-2, and JTAI-3) against PAI-1 for the same epitope on PAI-1 was tested as previously described. Peroxidase-coupled MoAbs were prepared by coupling the antibodies to horseradish peroxidase using m-periodate by the method of Nakane and Kawaoi.

Clinical groups. DIC patients (n = 58) in this study were separated into two groups: groups with (n = 15) or without APL (n = 43). The presence of DIC was diagnosed for each patient by marked reductions in platelet counts, fibrinogen, antithrombin III (except in APL without sepsis), plasminogen, and α2-plasmin inhibitor levels and elevated levels of fibrin degradation products. In this study, DIC patients with APL and without sepsis (n = 13) were carefully chosen and separated from the two cases with severe sepsis (see Fig 5, lanes g and h). The DIC patient group without APL included 23 patients with leukemia, 12 patients with lung cancer, 6 patients with gastric cancer, and 2 patients with biliary tract infection. Thirty-two patients had either high- or low-grade fever. A sepsis group without DIC included five patients with malignant tumor, two with respiratory tract infection, and five with peritonitis.

Plasma. Blood was collected from the antecubital veins of healthy volunteers and patients into 0.1 vol 3.8% sodium citrate with or without theophylline (final 0.001 mol/L) and prostaglandin E1 (final 0.5 mg/L) with a 30-second hand cuff at about 9 AM. The collected blood was immediately cooled with ice water and centrifuged at 2,000g for 20 minutes at 4°C to prepare platelet-poor plasma (PPP). All plasma was stored at −80°C until use. Gel-filtered platelets and platelet-rich plasma were prepared as described. PAI-1–depleted plasma was prepared from pooled healthy volunteers’ plasma by passage through rabbit anti–PAI-1 IgG-immobilized Sepharose (5 mg IgG/mL Sepharose), which had been equilibrated with a mixture of 0.9 vol 0.15 mol/L NaCl and 0.1 vol 3.8% sodium citrate. Single time point plasma samples were taken from patients with DIC.

Plasma clot lysis induced by MoAb against α2-plasmin inhibitor (α2-PI). Plasma was supplemented with 2 μmol/L Glu-plasminogen and 1.2 μmol/L of anti–α2-PI MoAb JTP-1, which induces spontaneous plasma clot lysis. α-Thrombin (final 0.1 U/mL) and CaCl2 (final 0.018 mol/L) were added, and the solution was allowed to clot for 15 minutes at 37°C in a glass tube. The clots were wound onto a bamboo stick and squeezed to remove as much fluid as possible. Clots were subsequently resuspended in the same sera. Fibrinolysis was monitored by measuring the amount of fibrin degradation products in the supernatant after 3 hours of incubation at 37°C using counter-immunoellectrophoresis as described. The amount of FDP before incubation was subtracted from this value.

Solid-phase ELISA for total PAI-1. Polyvinyl chloride microtiter plates were coated with a mixture of two MoAbs against PAI-1 (10 μg/mL JTAI-1 and JTAI-2) for 16 hours at 4°C as described. The plates were emptied and the wells were treated for 2 hours at 25°C with 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4 (TBS buffer) containing 1% BSA. After washing two times with TBS containing 0.05% Tween 20 (TBS-Tween) and 0.1% BSA, samples were diluted in the same buffer and aliquots were added to the coated wells. When samples were plasma, the samples were diluted with the same buffer four times and the standard curves were constructed with various concentrations of purified PAI-1 diluted with a final 25% PAI-1 immunodepleted plasma. Variability due to clotting was assessed by the commercial ELISA kit Elisaclon PAI-1 (Biopool AB, Umea, Sweden).

MoAbs against PAI-1, using either t-PA/PAI-1 complex or latent PAI-1 as an antigen, were raised by standard methods using BALB/c mice and the mouse myeloma cell line P3U1 as described. The dissociation constants (kd) of 125I-labeled MoAbs for the latent PAI-1 and t-PA/PAI-1 complex were assessed by a solid-phase assay as described, according to the Frankel and Gerhard method. The competition between MoAbs (JTAI-1, JTAI-2, and JTAI-3) against PAI-1 for the same epitope on PAI-1 was tested as previously described. Peroxidase-coupled MoAbs were prepared by coupling the antibodies to horseradish peroxidase using m-periodate by the method of Nakane and Kawaoi.

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monitored at 25°C on an ELISA analyzer ETY-96 (Toyo Sokki, Tokyo, Japan) for 30 minutes.

**Solid-phase ELISA for t-PA/PAI-1 complex.** The ELISA for t-PA/PAI-1 complex in samples was performed basically as described above using JTAI-1 as the coating antibody and peroxidase-anti-t-PA MoAb JTA-1. To assess the level of active PAI-1 antigen in a sample, single-chain t-PA (final 75 ng/mL) and aprotinin (final 500 U/mL) were added to the sample and the mixture was incubated for 30 minutes at 37°C. After incubation, samples were diluted four times with TBS-Tween containing 0.1% albumin, and differences due to interference of plasma proteins were adjusted with PAI-1-depleted plasma added just before ELISA. The quantity of active PAI-1 was calculated from the amount of t-PA/PAI-1 complex formed after correction for the amount of complex in the original sample and assuming a 1:1 stoichiometry.

**SDS-PAGE.** SDS-PAGE in slab gels was performed using resolving gels of 10% acrylamide and stacking gels of 4% according to Weber and Osborn. Molecular weight standards were obtained from Bio-Rad. To observe the profile of PAI-1 from plasma, plasma was immunoabsorbed with anti-PAI-1 polyclonal antibody immobilized on Sepharose. The column was eluted with sample buffer for SDS-PAGE containing 2% SDS, and samples were fractionated by SDS-PAGE. The gel was processed for reverse fibrin autography or Western blotting. Western blotting of the samples was performed after electrical transfer to nitrocellulose, using anti-PAI-1 IgG, as previously described.

**Miscellaneous.** Human umbilical vein endothelial cells were isolated from human umbilical cord veins by the method of Jaffe et al and were cultured as described. Plasma concentrations of scu-PA were measured basically as described previously by ELISA using anti-urokinase MoAb JS-1 (final 10 μg/mL) as a coating antibody, peroxidase MoAb JS-2 as a second antibody, and purified scu-PA as the standard urokinase-type plasminogen activator (u-PA). This ELISA measures the amount of single-chain u-PA, two-chain u-PA that was activated by plasmin, and u-PA complexed with PAI-1 by using the same standard curve for free scu-PA (data not shown). Purified PAI-1 was reactivated with either 8 mol/L urea or 4 mol/L guanidine hydrochloride at pH 5.5 for 30 minutes at 37°C, and dialyzed for 12 hours at 4°C against 0.05 mol/L sodium acetate pH 5.5 containing 0.1 mol/L NaCl and 0.05% Tween 20 and for 2 hours at 4°C against TBS-Tween pH 7.4. PAI activity in a sample was assessed by the method as described (conventional functional assay of PAI) using single-chain t-PA, plasminogen (1 μmol/L), CNBr-digested fibrinogen (1 μmol/L), and S-2251 (Kabi Diagnostica, Stockholm, Sweden) (0.5 mmol/L). One unit of PAI is defined as the amount of PAI that neutralizes 2 ng of single-chain t-PA in 10 minutes at room temperature. MoAbs against PAI-1 were radiiodinated using lactoperoxidase-glucose oxidase and Na I 125I (20 Ci/mg) (New England Nuclear, Boston, MA). The labeled MoAbs JTAI-1, -2, -3, -4, and -5 had specific activities of 6.8 × 105, 7.2 × 103, 7.7 × 105, 8.3 × 103, 6.3 × 105, and 8.8 × 100 cpm/μg, respectively. The concentration of α2-PI/plasmin complex in plasma was measured using the ELISA kit TD-80C (Teijin Co, Tokyo, Japan).

**RESULTS.**

Coagulation and fibrinolysis in DIC patients with APL. DIC associates frequently with APL even in the absence of severe infection and results in a high mortality from hemorrhage, especially intracranial bleeding. Although thrombin generation was reported in DIC with APL neither activity and antigen levels of protein C nor antithrombin III levels decreased in our DIC patients with APL in the absence of sepsis. Because sepsis is one of the major triggers of DIC, we carefully selected DIC patients for those with APL and without sepsis.

As shown in Table 1, the α2-PI antigen and fibrinogen levels in cases of DIC with APL were significantly lower and the level of FDP was significantly higher than observed in the group of DIC patients with other diseases. These results suggested that a marked increase in secondary fibrinolysis occurs in DIC patients with APL. However, in these patients the consumption of antithrombin III and protein C, and the increase in PAs and plasmin-α2-PI complex levels were not as great as those found in the control DIC group.

**Plasma clot lysis induced by an MoAb against α2-PI.** In DIC, "secondary fibrinolysis" that occurs on the fibrin clots plays a role not only in the removal of formed clots but also in the prevention of fibrin deposition. To test the capacity for initiation of fibrinolysis in these patients, we observed secondary fibrinolysis by adding a small amount of thrombin and 1.2 μmol/L MoAb against α2-PI (MoAb JTAI-1) as described in Materials and Methods. This MoAb induces complete spontaneous clot lysis when incubated at 37°C for 10 hours after clotting, as described. Because the concentration of plasminogen in plasma affected this spontaneous clot lysis and this effect leveled off in the presence of more than 2 μmol/L plasminogen (data not shown), we added 2 μmol/L Glu-plasminogen to each plasma sample before clotting. As shown in Fig 1, the ratios of the amount of fibrin degradation products produced after 3 hours of incubation at 37°C relative to the antigen level of t-PA were significantly different in patients with different disease states. The ratios in patients with septicemia were remarkably low and

| Table 1. Various Parameters Relating to Coagulation and Fibrinolysis in DIC Patients |
|---------------------------------|---------------------------------|-----------------|-----------------|
|                               | DIC (APL-) [N = 43]            | DIC (APL+) [N = 13] | Sepsis (N = 12) | Normal (N = 40) |
| AT III (%)                     | 68.8 ± 26.4                    | 109.2 ± 12.3      | 98.6 ± 10.3     | 100 ± 10        |
| Fibrinogen (mg/100 mL)         | 277.8 ± 158.4                  | 80 ± 40.7         | 414.1 ± 152.0   | 250 ± 50        |
| FDP (μg/mL)                    | 78.2 ± 68.1                    | 217.1 ± 206.0     | <10             | <10             |
| α2-PI (μg/mL)                  | 76.3 ± 21.2                    | 42.9 ± 11.2       | 95.5 ± 12       | 100 ± 10        |
| PIC (μg/mL)                    | 5.2 ± 6.3                      | 2.2 ± 3.1         | <0.5            | <0.5            |
| u-PA (ng/mL)                   | 2.9 ± 1.6                      | 3.3 ± 2.1         | 2.6 ± 1.7       | 2.2 ± 1.8       |
| t-PA (ng/mL)                   | 78.2 ± 68.1                    | 11.0 ± 6.4        | 19.3 ± 15.0     | 8.92 ± 7.89     |

Plasma was analyzed as described in Materials and Methods. Values are mean ± SD.

Abbreviations: APL , without APL; ATIII, antithrombin III; FDP, fibrinogen and/or fibrin degradation products; PIC, plasma α2-PI complex; u-PA, urokinase-type plasminogen activator.
those in DIC patients without APL were relatively low in comparison with the level of t-PA. Interestingly, the ratios in DIC plasma with APL were not lower than those in normal plasma. Because this assay was intended to observe the regulation of the fibrinolysis in the initiation step by neutralizing antiplasmin activity, we examined the antigen.

Development of ELISA for total PAI-1 and t-PA/PAI-1 complex. We selected three MoAbs against PAI-1 (JTAI-1, JTAI-2, and JTAI-3) whose kds were similar for free PAI-1 or the t-PA/PAI-1 complex: JTAI-1, \( kd = 9.7 \times 10^{-9} \) mol/L for latent PAI-1 and \( 4.8 \times 10^{-9} \) mol/L for complex; JTAI-2, \( kd = 7.8 \times 10^{-9} \) mol/L and \( 11.2 \times 10^{-9} \) mol/L; and JTAI-3, \( kd = 4.7 \times 10^{-9} \) mol/L and \( 12.3 \times 10^{-9} \) mol/L, respectively. Furthermore, these three MoAbs did not compete with one another for binding to free PAI-1 or t-PA/PAI-1 complex in a competition assay (data not shown).

To determine whether an ELISA using any pair of the MoAbs could detect both t-PA-unbound PAI-1 and the t-PA/PAI-1 complex with a similar efficiency, standard curves were constructed using PAI-1-depleted pooled normal plasma supplemented with either purified PAI-1 or t-PA/PAI-1 complex. In any combination of two MoAbs, the reactivities toward free PAI-1 and the t-PA/PAI-1 complex were at least one and a half times different (data not shown). Therefore, we used a mixture of JTAI-1 and JTAI-2 as the coating antibodies and peroxidase-labeled JTAI-3 to develop an assay for total PAI-1 antigen. Using this system, free PAI-1 and t-PA/PAI-1 complex were recognized equivalently when diluted in normal PAI-1-depleted plasma (Fig 2A). However, perhaps due to the double antibody-coating, this assay was not so sensitive (about 0.6 ng/mL). Because plasma had to be diluted at least 32 times to minimize the interference of plasma proteins and the interdilution coefficient of variation of four serial dilutions (4, 8, 16, and 32 times) of 20 different samples was 58%, the samples were diluted with PAI-1-depleted plasma. Under these modified conditions, the interdilution coefficient of variation of 20 different samples was 5.1%. When adding purified latent PAI-1 or t-PA/PAI-1 complex to PAI-1-depleted plasma of final concentrations of 10, 50, or 100 ng/mL, recoveries from this assay were 110% ± 5%, 95% ± 4%, and 101% ± 6% for latent PAI-1 and 95% ± 7%, 102% ± 7%, and 90% ± 4% for t-PA/PAI-1 complex, respectively (mean ± SD, \( n = 3 \)). Inter-assay and intra-assay coefficients of variation were 7.9% and 5.2% (\( n = 10 \)), respectively.

To develop an ELISA for the t-PA/PAI-1 complex, JTAI-1 was used as the coating antibody and peroxidase-labeled anti-t-PA MoAb (JTA-1) was used to detect t-PA antigen as it recognizes both free t-PA and the t-PA/PAI-1 complex with a similar efficiency. Figure 2B illustrates a dose-response curve of purified t-PA/PAI-1 complex diluted to varying concentrations in normal PAI-1-depleted plasma. In this sandwich ELISA, addition of a greater than 50-fold mol/IL excess of free latent PAI-1 to the samples just before assay did not interfere with the measurement of t-PA/PAI-1 complex with a similar efficiency. Inter-assay and intra-assay coefficients of variation were 6.8% and 4.9%, respectively (\( n = 10 \)).
Fig 2. Dose-response curves of total PAI-1 and t-PA/PAI-1 complex and correlation between PAI activity and active PAI-1 antigen. (A) Various concentrations of purified latent PAI-1 (○) or t-PA/PAI-1 (●) complex were added to normal PAI-1-depleted plasma and their differential reactivities were determined by the ELISA for total PAI-1 as described in Materials and Methods. The abscissa shows the amount of latent PAI-1 and calculated PAI-1 in the complex assuming a 1:1 complex between t-PA and PAI-1. (B) Various concentrations of purified t-PA/PAI-1 complex were added to normal PAI-1-depleted plasma and concentrations of complex were determined as described in Materials and Methods. The data represent the mean of three experiments. (C) Urea- or guanidine-activated PAI-1 samples (two each) were diluted with either TBS-Tween containing 0.1% BSA (○) or PAI-1-depleted plasma (●) as described in Results, and active PAI-1 levels were determined by a conventional method (ordinate) and by our modified ELISA (abscissa). One unit of PAI is defined as the amount of PAI that neutralizes 2 ng of single-chain t-PA. (D) Ten sepsis patient plasmas without DIC, 16 DIC patient plasmas, and 14 normal plasmas were collected as described in Materials and Methods, and active PAI-1 levels were determined by ELISA as described above.

**Effect of complexation of PAI-1 to vitronectin and the decay of active PAI-1 to the latent form on the measurement of PAI-1 antigen.** Because plasma vitronectin binds to active PAI-1 and apparently stabilizes PAI-1 activity twofold to threefold in blood, it was critical to know to what degree the ELISA for total PAI-1 measures vitronectin-complexed PAI-1. Furthermore, because there may be free active and latent PAI-1 in biologic fluids, it was also important to test the reactivities of the ELISA toward active and latent PAI-1. To these ends, total PAI-1 antigen and the time-dependent decay of active antigen levels were determined in the presence or absence of exogenous vitronectin (Fig 3A).
using either form of PAI-1, although active PAI-1 in the
conditioned media was 8.3% ± 2.1% (mean ± SD, n = 3)
of total PAI-1 antigen. The level of active PAI-1 antigen
decreased gradually in the absence of vitronectin (half-life:
1.5 hours), while in the presence of vitronectin the half-life
was prolonged (3 hours) at 37°C. By using a sandwich
immunoradiometric assay we could detect complexes of
PAI-1 and vitronectin in the presence of exogenous vitronec-
tin. In contrast, total PAI-1 antigen did not change even if
activated PAI-1 decayed into the latent form, or if activated
PAI-1 complexed with vitronectin (Fig 3A).

Because PAI-1 in plasma or platelet releasates were
supposed to bind to VN, the effect of decay of active PAI-1
in these samples on the measurement of total PAI-1 antigen
by this assay was determined at 37°C. The specific activity
of PAI-1 from platelet releasates decayed with a half-life of 3
hours, whereas total PAI-1 antigen did not change (Fig 3B).
The releasate from the different methods for aggregating
platelets did not show any distinct differences in their rates
of loss of PAI-1 activity. At 37°C, PAI-1 activity also
decayed at a similar rate (half-life: 3 hours) for normal
plasma, DIC patient plasmas, or for normal citrated whole
blood (Fig 3C).

Evaluation of plasma PAI-1 in patients. Total PAI-1
levels in plasma from DIC patients and from patients with
sepsis were higher than those from normal controls (Fig
4A). However, the specific activity of PAI-1 (ratio of active
PAI-1 to t-PA unbound PAI-1) in plasma from DIC
patients, especially those with APL, was greatly reduced in
comparison with that observed from sepsis patients or
normal controls (Fig 4B). The level of t-PA–unbound
PAI-1 was calculated by subtracting the value of 5/12 ×
t-PA/PAI-1 complex) from the total PAI-1 concentration.
During DIC, platelets are continuously activated by throm-
bin or other agonists in vivo and released PAI-1 from
platelets may contribute to the increase in PAI-1 antigen.
The specific activity of platelet PAI-1 determined by our
ELISA method was about 6%, a result almost identical to
that described by Declerck et al (about 5%). These results
suggest that an increasing level of platelet PAI-1 released
into the plasma may be one explanation for the observed
low PAI-1 specific activities in DIC patients. However,
because the APL patients had low platelet counts and an
extremely low PAI-1 specific activity, the profiles of PAI-1
antigen in patient plasmas were examined by Western
blotting (Fig 5). As shown in Fig 5, lane f, PAI-1 appeared
to be cleaved, presumably by some protease(s) present in
the patient plasma. Additionally, in DIC associated with
APL and sepsis (Fig 5, lane h), the high molecular weight
(MW, ~110,000) form of PAI-1, which also reacted with
anti-t-PA antibody by Western blotting, and degraded
forms of PAI-1 were present as well as an apparent overall
increased level of PAI-1 antigen.

DISCUSSION

APL has been characterized by proliferation of morpho-
logically abnormal promyelocytes and a severe bleeding
diathesis. The bleeding diathesis is due mainly to DIC
initiated by release of procoagulant activity from abnormal
granules in the promyelocytes. Thrombin generation and
Fig 4. Total PAI-1 antigen and specific activity of PAI-1 in DIC patient plasma. The levels of total PAI-1 antigen, active PAI-1 antigen, and t-PA/PAI-1 complex were measured as described in Materials and Methods. Patient plasmas: DIC without APL (●), DIC with APL without sepsis (■), sepsis without DIC (□), and normal volunteer plasma (▲). Values are mean ± SD.

Fig 5. PAI-1 antigen in DIC patient plasma. Conditioned media of cultured human endothelial cells (a), and plasma for normal (b), sepsis (c), DIC with sepsis (d), and DIC with lung cancer (e), DIC with APL in the absence of sepsis (f), and DIC with APL in the presence of sepsis (g and h). Plasma samples were immunoadsorbed by anti–PAI-1 Sepharose and the eluate was fractionated and subjected to Western blotting using a mixture of three MoAbs (JTAI-1, -2, and -3) as described in Materials and Methods.
inherently active PAI-1 from the conditioned media of HUVEC, we cannot exclude the possibility that the relatively low specific activity of the PAI-1 in the conditioned media may lead to the invisibility of an existing differential reactivity of this assay between inherent active PAI-1 and the other forms of PAI-1. A number of assay systems have been developed that estimate PAI-1 activity in biologic fluid. For example, measurement of residual PA activity, either directly or by measuring plasminogen activating activity in the presence of fibrin fragments after adding a fixed amount of PA to the samples, is one of the typical PAI activity assays. However, these assays cannot discriminate PAI-1 activity from other PA inhibitors, in particular PAI-2 activity during pregnancy and PAI-3 in the presence of heparin. We can measure the concentration of active PAI-1 antigen specifically by measuring t-PA/PAI-1 complex after adding a fixed amount of t-PA to the samples. A similar approach for the measurement of active PAI-1 has also been described by several groups.

Any measurement of PAI-1 levels in biologic fluids must rely on an appropriate and stable standard material for accurate determinations. For functional PAI-1 assays, the choice of a standard is particularly complicated because it is a trace plasma protein whose activity decays into latency. In addition, PAI-1 is present primarily in the latent form in most conditioned media from cultured cells and in platelets. The current assay measures active PAI-1 by virtue of its ability to bind a known amount of added t-PA, and the standard was purified t-PA/PAI-1 complex. This design obviates the need to activate latent PAI-1 for a standard, and the complex is inherently more stable than t-PA–unbound active PAI-1 because the complex is acyl-enzyme complex. Labeled t-PA/PAI-1 complex dissociated somewhat (from about 5% to 35%) under nonreduced conditions according to the size of the gel and the time of the electrophoresis by Laemmli's SDS-PAGE method, and this may be due to nucleophilic attack on the acyl-enzyme complex by the positively charged Tris ion at high pH as described. However, less than 5% of the complex dissociated by the Weber and Osborn SDS-PAGE method or by gel-filtration chromatography using a TSK-GEL G3000 SW column and a CCP-8010 HPLC system (Toyo Soda, Tokyo, Japan) after 18 hours at room temperature or 2 months of storage at −20°C (data not shown). We cannot rule out the possibility that a small amount of degraded t-PA/PAI-1 complex may be generated by adding t-PA to the samples. In fact, the slope of the calibration curve between active PAI-1 levels measured with the current assay and those with the conventional method in a purified system (Fig 2C: slope = 0.65) or in patient plasmas (Fig 2D: slope = 0.57) was smaller than the estimated one (slope = 0.7) (Fig 2C). However, the correlation was satisfactory enough, so that we used the present modified ELISA method to analyze the total PAI-1 antigen, t-PA/PAI-1 complex, and active PAI-1 antigen in the patient plasmas.

The PAI-1 antigen levels increased in DIC patients and in patients with septicemia (Fig 4A), and the FP ratios in these patients were lower than those in normal controls (Fig 1). These results may suggest the retardation of clot lysis in vivo by the elevated PAI-1 in these diseases. The ratio in DIC patients was higher than that in patients with septicemia. This difference may be explained by the fact that the specific activity of PAI-1 in patients with DIC decreased significantly in comparison to patients with septicemia or in normal controls (Fig 4B). In DIC patients the PAI-1 antigen levels may increase because of release from platelets by thrombin and other agonists, or increased production by endothelial cells after endotoxin, interleukin-1, or ischemic stimulation. In general, the plasma from a DIC patient without APL contained a small amount of degraded PAI-1 as observed on Western blots. However, the majority of the PAI-1 migrated as an intact Mr 50,000 band. Because PAI-1 in the DIC plasma decayed into the latent form at a rate similar to PAI-1 in normal plasma (Fig 3C), it is unlikely that the low specific activity results from an instability of the active PAI-1. However, it is possible that this inactive PAI-1 originated from activated platelets, assuming increased platelet activation during DIC. Although the level of total PAI-1 antigen in DIC patient plasma with APL was higher than that in normal controls, the FP ratio was similar to that in normal control. This may be due to the low specific activity of PAI-1 in DIC patient plasma with APL (Fig 4B).

In DIC patient plasma with APL, marked hyperfibrinolysis in vivo was suggested by the increase of FDP and the decrease of fibrinogen and a2-PI activity, and the level of plasmin/a2-PI complex did not increase (Table 1). Because the antigen levels of PAs were not greatly elevated, hyperfibrinolysis in DIC with APL may be due to at least two factors: (1) proteolytic cleavage of fibrinogen, a2-PI, and PAI-1 (Fig 5) by leukocyte elastase or other proteases from abnormal promyelocytes; and (2) increased secondary fibrinolysis. There are several reports of decreased levels of either a2-PI or PAI-1, and the patients had bleeding tendencies. In these cases, the antigen levels of t-PA were normal and treatment of these individuals with epsilon-aminocaproic acid was effective. This suggestion is further supported by the degraded forms of PAI-1 observed in plasmas from DIC patients associated with APL and sepsis (Fig 5, lanes g and h). In one case (Fig 5, lane h), the combined effects of endotoxin and APL protease on the marked increase of t-PA and PAI-1 and proteolysis of PAI-1 might result in the appearance of t-PA/PAI-1 complex and the degraded forms of PAI-1.

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The specific activity of plasminogen activator inhibitor-1 in disseminated intravascular coagulation with acute promyelocytic leukemia

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