reported previously.[15] Briefly, the presence of underlying disease—such as infection and malignancies—specific clinical conditions (bleeding symptoms, organ dysfunction), and results of laboratory examinations (platelet counts, prothrombin time, fibrinogen, fibrin degradation products) were quantified based on score. If the score was 7 or more, the diagnosis of DIC was made. In patients with hematologic malignancy, scores on the bleeding symptom and platelet counts were excluded, and the diagnosis of DIC was made if the total score was 4 or more.

The diagnosis of sepsis was made according to the guidelines of the Society of Critical Care Medicine Consensus Conference Committee.[16] Briefly, patients had to meet at least 3 of the 4 criteria for systemic inflammatory response and had to have a known infection or a suspected infection, as evidenced by one or more of the following: bacteremia, pathologic microorganisms or white blood cells in a normally sterile body fluid such as urine or joint fluid; purulent sputum; radiographic evidence of pneumonia; clinical signs associated with high risk for infection (eg, cholangitis, peritonitis) or increased levels of endotoxin, β-D-glucan, or *Candida* antigen.

Thirty-nine patients with DIC were shown to have bacteremia, as evidenced by their blood cultures. Twelve patients, whose bacteremia was not evidenced by blood culture, had increased levels of endotoxin, β-D-glucan, or *Candida* antigen. Twenty-eight patients who were negative for bacteria in blood culture or who did not have increased levels of endotoxin, β-D-glucan, or *Candida* antigen, had pneumonia as evidenced by radiography, 11 patients had urinary tract infection, 4 patients had wound infection during postoperative periods, 1 patient had biliary tract infection, 1 patient had bacterial arthritis, 1 patient had bacterial osteomyelitis, and 12 patients had suspected respiratory infection with the presence of pathogenic microorganisms, such as methicillin-resistant *Staphylococcus aureus* in sputum cultures.

Citrated platelet-poor plasma samples were prepared and stored at −80°C until use. Blood was also drawn from 12 healthy volunteers (7 men, aged 25-53; 5 women, aged 25-48) for the preparation of normal pooled plasma. Laboratory analyses of patients' blood were performed by the standard methods using automated analyzers. Complete blood cell counts, serum creatinine (normal range, 35-97 μM [0.4-1.1 mg/dL]), serum bilirubin (normal range, 3-21 μM [0.2-1.2 mg/dL]), aspartate aminotransferase (AST; normal range, 8-35 IU/L), alanine aminotransferase (ALT; normal range, 5-40 IU/L), serum albumin (normal range, 39-51 g/L [3.9-5.1 g/dL]), and C-reactive protein (CRP; normal range, less than 5 mg/L [0.5 mg/dL]) were measured in this study.

Determination of ADAMTS13 antigen and activity levels

The human *ADAMTS13* cDNA used in this study was described previously.[5] Human ADAMTS13 was expressed in human embryo kidney 293 cells stably transfected with pCAG-ADAMTS13 Neo and was purified. Murine monoclonal antibodies (mAbs) to human ADAMTS13 were generated by the standard method[17] after immunization of BALB/c mice with recombinant human ADAMTS13. Two mAbs, WH10 and WH2-22-1A, were selected for ELISA, which was shown to bind to the third TSP-1 motif and to the disintegrin domain of ADAMTS13 by the binding study to recombinant ADAMTS13 mutants, respectively.[5,14,18] WH10 (2 μg/mL) was used for microtiter plate coating (Maxi Sorp plate; Nalge Nunc International, Rochester, NY). After blocking with 1% casein, plasma samples from healthy subjects and patients were diluted in phosphate-buffered saline, pH 7.2/0.1% casein, and then incubated in WH10-coated plates. ADAMTS13 bound to the microtiter plates was detected by peroxidase-conjugated WH2-22-1A. Purified recombinant ADAMTS13 was used as the standard to determine ADAMTS13 antigen levels in normal plasma. The ADAMTS13 level in each patient's plasma was expressed as the percentage of that in normal pooled plasma. ADAMTS13 activity levels in plasma were measured according to the previously described method.[14] Briefly, 10 μL plasma was mixed with purified VWF (1 μg) in 100 μL reaction buffer (5 mM Tris [pH 8.0]/1.5 M urea/10 mM $BaCl_2$/0.4 mM Pefabloc SC [Roche Diagnostics, Mannheim, Germany]) at 37 °C for 24 hours. Reaction was terminated by the addition of 10 μL of 500 mM EDTA, pH 8.0.[14] Portions of samples were subjected to 1.4% sodium dodecyl sulfate–agarose gel electrophoresis to determine the extent of VWF degradation. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and VWF multimers were detected by peroxidase-labeled rabbit anti–human VWF antibodies (Dako, Glostrup, Denmark).[12-14]

Quantification of molecular markers of DIC

Plasma levels of fibrin degradation products (FDPs) were quantified with commercial kits (Roche Diagnostics, Tokyo, Japan) used for laboratory examinations. Given that the quantification of free thrombin concentration in plasma is technically difficult, we used ELISA (Sysmex, Kobe, Japan) to quantify plasma levels of thrombin/antithrombin III (TAT) complexes. Similarly, plasma levels of plasmin/α2 plasmin inhibitor complexes (PICs) were measured using ELISA with commercial kits (Sysmex) used for laboratory examinations. Plasma plasminogen activator inhibitor 1 (PAI-1) levels were quantified by the latex photometric immunoassay by using a commercial kit (Mitsubishi Kagaku Iatron, Tokyo, Japan), as described previously.[19] The granulocyte elastase digests of cross-linked fibrin (granulocyte elastase–dependent fibrin degradation products [E-XDPs]) were measured by the automated latex photometric immunoassay using IF-123 monoclonal antibody, which is specific for the fibrin fragment D species generated by granulocyte-elastase digestion.[20] Monoclonal antibody IF-123–bound latex particles (Mitsubishi Kagaku Iatron) were used for the assay. A 2.4-μL aliquot of sample plasma was mixed with 32 μL latex reagents in 250 μL Tris-buffered saline, and then absorbance changes were analyzed with an automated analyzer for latex photometric immunoassay (model LPIA-NV7; Mitsubishi Kagaku Iatron). The standard E-XDP was purified according to the method of Kohno et al.[20] The normal range of plasma E-XDP levels is less than 3 U/mL.

Effect of granulocyte elastase on ADAMTS13

Recombinant ADAMTS13 (250 nM) was incubated in 20 μL Tris-buffered saline, pH 7.4, in the absence or presence of granulocyte elastase (Elastin Products, Owensville, MO) at 5 nM and 50 nM. Aliquots (5 μL each) were harvested after incubation at 37°C for 5, 15, and 30 minutes. The reaction of each aliquot was terminated by addition of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS. Samples were then analyzed by SDS-PAGE followed by Western blotting with anti-ADAMTS13 monoclonal antibody WH2-22-1A and peroxidase-labeled anti–mouse IgG.

Detection of ADAMTS13 molecular forms in plasma

Western blot analysis of ADAMTS13 in plasma by mAb WH2-22-1A was performed after immunoprecipitation with anti-ADAMTS13 polyclonal antibody immobilized to protein G-Sepharose.

Analysis of VWF multimers in patient plasma

VWF multimers in patient plasma were analyzed by SDS-agarose gel electrophoresis according to the method described previously.[12-14]

Results

ELISA for ADAMTS13

We generated mAbs against recombinant human ADAMTS13 and used them to develop an mAb-based ADAMTS13 ELISA. To determine the specificity of this assay, plasma obtained from a patient with Upshaw-Schulman syndrome was mixed with normal plasma at various ratios, and the ADAMTS13 activity and antigen levels were measured. As shown in Figure 1, ADAMTS13 activity and ADAMTS13 antigen levels in the plasma of the patient with Upshaw-Schulman syndrome were less than 1%, and the ADAMTS13 antigen level in the patient plasma increased linearly in parallel with the ADAMTS13 activity in the presence of increasing amounts of normal plasma. The correlation coefficient

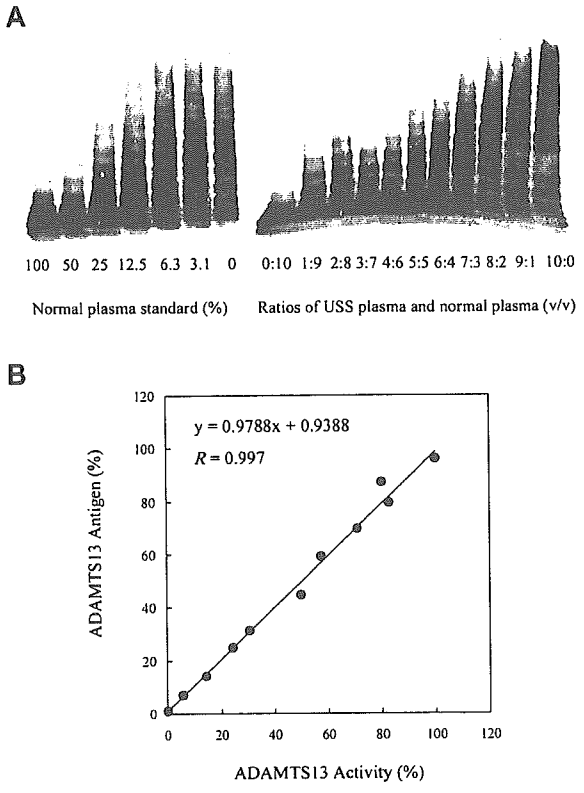

Figure 1. Analysis of ADAMTS13 activity and antigen levels in plasma of patients with Upshaw-Schulman syndrome. ADAMTS13 activity and antigen levels were determined in the plasma of a patient with Upshaw-Schulman syndrome (USS) mixed with normal pooled plasma at various ratios. (A) Result of ADAMTS13 activities in the plasma of the USS patient mixed with normal plasma at various ratios (0:10-10:0). (B) Correlation of ADAMTS13 activity and antigen levels in these samples.

between ADAMTS13 antigen and ADAMTS13 activity was 0.997. The ADAMTS13 level in normal pooled plasma was 1.57 μg/mL when recombinant human ADAMTS13 was used as the standard. The calibration curve was linear ($r = 0.999$), and the ELISA could distinguish absorbance changes of ADAMTS13 at 0.3% of the normal plasma level from ADAMTS13-depleted plasma. Interassay variability in samples containing 50% and 100% of ADAMTS13 were 7.9% and 5.2%, respectively.

ADAMTS13 levels in disease states

ADAMTS13 antigen and activity levels in the plasma of patients with sepsis-induced DIC or TTP were studied (Figure 2). The

Table 1. Correlation between the ADAMTS13 levels and molecular markers of DIC in patients with sepsis-induced DIC

	ADAMTS13*		
	Activity	Antigen	Activity-antigen ratio
Fibrinogen	−0.347	−0.244	−0.219
FDP	0.354	0.242	0.274
TAT	0.246	0.379	0.367
PIC	0.370	0.357	0.327
PAI-1	−0.230	−0.300	−0.006
Platelet	0.260	0.245	0.239
E-XDP	−0.399†	−0.404†	−0.229

n = 109 patients.
*Values are *rs* determined by Spearman rank correlation test.
†Statistically significant ($P < .01$).

correlation coefficient of ADAMTS13 antigen and ADAMTS13 activity was 0.80. As shown in Figure 2A, discrepancies between ADAMTS13 antigen levels and activity levels were observed in many samples. These discrepancies mainly were caused by the decreased level of specific ADAMTS13 activity compared with the ADAMTS13 antigen level. Some samples had higher specific activity of ADAMTS13. To explore the possibility that decreased levels of the ADAMTS13-specific activity correlated with disease states, Western blot analysis of ADAMTS13 molecular forms in patient plasma was performed. Low molecular–weight ADAMTS13 species were observed in DIC patient plasma by Western blotting (Figure 2B), indicating that proteolytic cleavage of ADAMTS13 could occur in this disease state. The recent report showed that ADAMTS13 could be digested in vitro by proteases such as thrombin and plasmin.[21] Because thrombin and plasmin can be generated in DICs, we tested the correlation between ADAMTS13 levels and molecular markers of coagulation and fibrinolysis. There was no correlation of ADAMTS13 activity, antigen, or specific activity level with levels of fibrinogen, FDP, TAT, PIC, PAI-1, or platelet counts (Table 1). We could only find a negative correlation between activity levels and antigen levels of ADAMTS13 and plasma levels of granulocyte elastase digests of fibrin (E-XDP) (Table 1; Figure 3A-B). Based on these results, we studied the effects of granulocyte elastase on ADAMTS13 in vitro. In accordance with previous reports, recombinant ADAMTS13 was determined to migrate at approximately 190 kDa by SDS-PAGE, followed by Western blotting.[14,21] As shown in Figure 3C, recombinant ADAMTS13 migrating at approximately 190 kDa was converted to the 120-kDa and 100-kDA fragments and finally to the approximately 40-kDa fragment on incubation with granulocyte

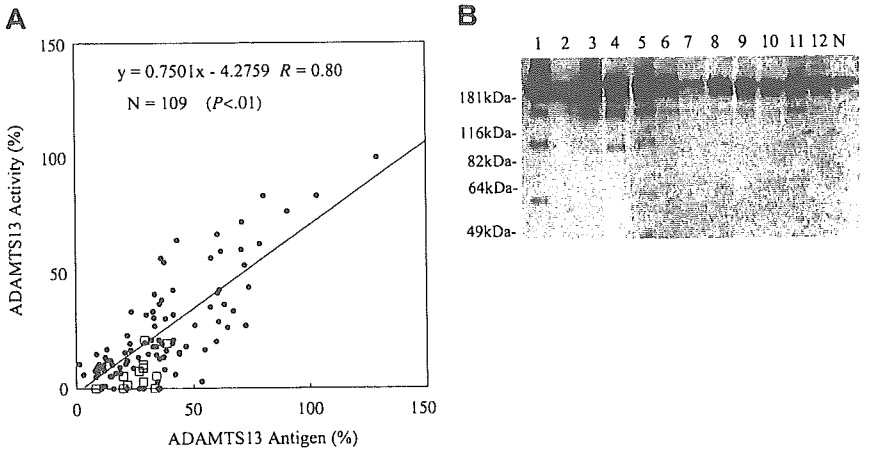

Figure 2. Analysis of ADAMTS13 activity, antigen, and molecular forms in plasma of patients with sepsis-induced DIC. (A) ADAMTS13 activity and antigen levels in the plasma of patients with sepsis-induced DIC were determined as described in "Patients, materials and methods." Samples (□) were subjected to immunoprecipitation followed by Western blotting to investigate the cleavage of ADAMTS13, as described in "Patients, materials, and methods." (B) Typical Western blot of degraded ADAMTS13 found in the patients' plasma indicated in panel A (□) is shown. Western blotting of ADAMTS13 antigen in normal pooled plasma (N) is shown as the control. ADAMTS13 molecules in normal plasma migrated at approximately 190 kDa.

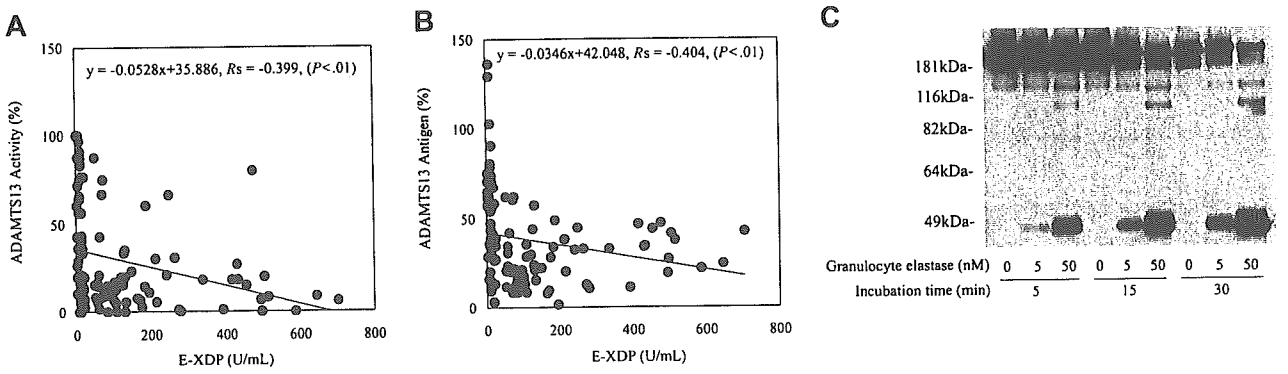

Figure 3. Correlation between the ADAMTS13 levels and the granulocyte elastase digests of cross-linked fibrin (E-XDP) levels in plasma of patients with sepsis-induced DIC and the effect of granulocyte elastase on ADAMTS13 in vitro. Correlations between the activity levels of ADAMTS13 and the plasma levels of granulocyte-elastase digests of fibrin (E-XDP) (A) and between the antigen levels of ADAMTS13 and the plasma levels of granulocyte-elastase digests of fibrin (E-XDP) (B) in patients with sepsis-induced DIC are shown. Values were analyzed by Spearman correlation coefficient by rank test. Recombinant ADAMTS13 was incubated with granulocyte elastase at 5 nM or 50 nM, and degradation of ADAMTS13 by granulocyte elastase was studied after the indicated time and analyzed as described in "Patients, materials, and methods" (C).

elastase in a dose-dependent and a time-dependent manner in vitro. A variety of lower molecular–weight ADAMTS13 fragments were detected in DIC patient plasma by Western blot (Figure 2B). According to the previous report, the ADAMTS13 fragments migrating approximately 150 to 170 kDa could be generated by thrombin.[21] ADAMTS13 fragments migrating approximately 120 kDa and 100 kDa in patient plasma might correspond to the granulocyte elastase digests of ADAMTS13. However, the 120-kDa ADAMTS13 fragment and the 100-kDa ADAMTS13 fragment could be generated by thrombin and plasmin, respectively.[21] It also is possible that thrombin-cleaved ADAMTS13 or plasmin-cleaved ADAMTS13 could be digested by granulocyte elastase or vice versa. These data may suggest that granulocyte elastase, together with other proteases (thrombin and plasmin plays a role in ADAMTS13 cleavage under certain pathologic conditions), may partially account for the decrease of the ADAMTS13-specific activity observed in DIC patients.

ADAMTS13 deficiency in disease states

ADAMTS13 antigen and activity levels in patient groups and in healthy subjects are shown in Figure 4. The plasma ADAMTS13 antigen and activity levels in untreated patients with TTP (no plasma exchange treatment, no fresh frozen plasma transfusion) were 13.5% ± 7.1% (range, 5.1%-19.6%) and 6.3% ± 5.7% (range, 0%-12.5%), respectively (idiopathic TTP 3, Upshaw-Schulman syndrome 1). Decreased levels of ADAMTS13 antigen and activity were observed in patients with sepsis-induced DIC compared with healthy subjects ($P < .01$) in this study, and severe decreases of ADAMTS13 activity and antigen levels were observed in patients with sepsis-induced DIC. Of the 109 patients with sepsis-induced DIC, decreases in ADAMTS13 activity levels (less than 5%) were found in 17 (15.6%) patients; clinical features and laboratory data of these patients are summarized in Table 2. Consciousness disturbance, thrombocytopenia, decreased hemoglobin levels, and increased LDH levels were commonly found in these patients. Clinical features were indistinguishable from those of patients with TTP, though patients with sepsis-induced DIC had evidence of the infection. Given that the highest ADAMTS13 activity level in patients with TTP without plasma exchange or blood transfusion was 12.5%, patients with sepsis-induced DIC were divided into 2 groups. One included patients with decreased ADAMTS13 activity levels (less than 20%; n = 51), and the other included patients with ADAMTS13 activity levels greater than 20% (n = 52). Patients with chronic renal failure before infection were excluded from this analysis. Patients were in severe condition; 25 (49.0%) of 51 patients in the former group and 35 (67.3%) of 52 patients of the latter group received transfusions of fresh frozen plasma, platelet concentrates, or both within 5 days of the determination of ADAMTS13 levels. This might have affected the activity and antigen levels of ADAMTS13.

Correlation between secondary ADAMTS13 deficiency and organ failure

Analyses of clinical and laboratory data showed that the patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) had elevated serum creatinine levels (Figure 5) that were

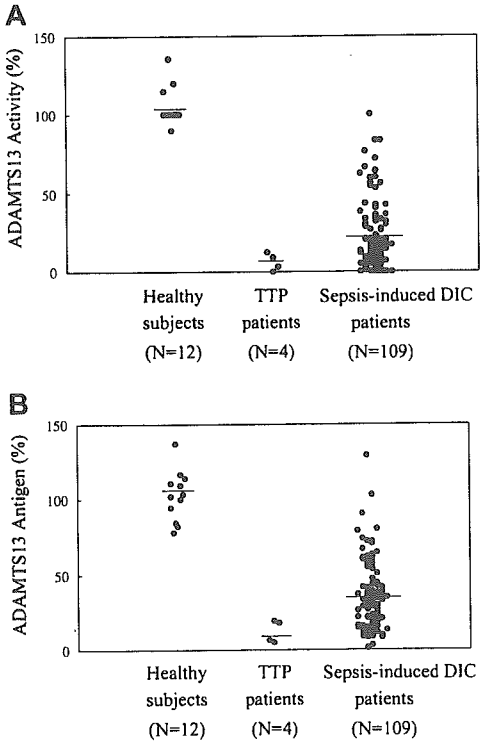

Figure 4. Plasma ADAMTS13 levels in patients and healthy subjects. ADAMTS13 activity levels (A) and antigen levels (B) of healthy subjects, patients with TTP (idiopathic TTP, 3; Upshaw-Schulman syndrome, 1) before plasma exchange treatment, and patients with sepsis-induced DIC (n = 109) are shown. Differences in the mean values (horizontal lines) between the healthy subject group and patient groups were statistically significant (nonrepeated measures ANOVA and Dunnett test; $P < .01$).

Table 2. Clinical profiles of patients with sepsis-induced DIC whose ADAMTS13 activity levels were lower than 5%

Characteristic	Value
Age, y	56.9 ± 21.3
Consciousness disturbance, no. (%)	8 (47.1)
Blood transfusion, no. (%)	11 (64.7)
ADAMTS13 antigen, %	25.5 ± 13.6
Creatinine, mg/dL	1.88 ± 2.06
Albumin, g/dL	2.2 ± 0.5
WBC count, cells/μL	11 200 ± 7 500
RBC count, × 10^4/μL	260 ± 86
Hemoglobin, g/dL	8.3 ± 2.0
Platelet count, × 10^4/μL	6.7 ± 5.3
LDH, IU/L	2481.3 ± 4107.8
CRP, mg/dL	18.11 ± 13.41

n = 17 patients.

Values for all categories except consciousness disturbance and blood transfusion are mean ± SD.

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

To convert albumin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert WBC count from cells per microliter to × 10^9 cells per liter, divide cells per microliter by 1000.

To convert RBC count from × 10^4 cells per microliter to × 10^{12} cells per liter, divide × 10^4 cells per microliter by 100.

To convert hemoglobin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert platelet count from × 10^4 platelets per microliter to × 10^9 per liter, multiply × 10^4 platelets per microliter by 10.

To convert CRP from milligrams per deciliter to milligrams per liter, multiply milligrams per deciliter by 10.

significantly higher than those in patients with ADAMTS13 levels higher than 20% (Table 3). The incidence of renal injuries in patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) was significantly higher than in patients with ADAMTS13 activity levels higher than 20% (Table 3). However, there were no differences in the incidence of liver dysfunction or serum levels of bilirubin, AST, and ALT among these groups (Table 3), suggesting that severe ADAMTS13 deficiency in these patients may be linked to the development of renal injuries. There was a significant difference in serum albumin levels between both groups, suggesting that the decrease of ADAMTS13 activity and antigen levels in patients was at least partially caused by reduced synthesis in the liver.

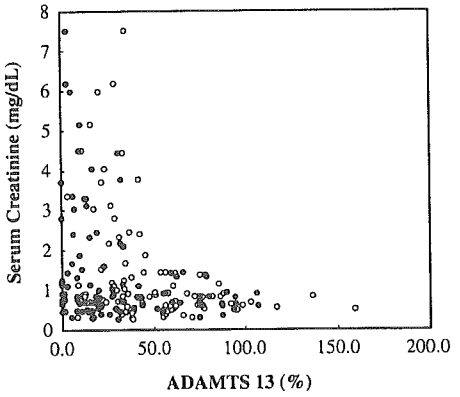

Figure 5. Correlation between the plasma ADAMTS13 levels and the serum creatinine levels. Correlation between serum creatinine levels and ADAMTS13 activity (●) levels or antigen (○) levels in patients with sepsis-induced DIC is shown (n = 103). Patients with a history of chronic renal failure were excluded from the study.

Table 3. Correlation between ADAMTS13 levels and organ injury in patients with sepsis-induced DIC

	ADAMTS13 activity less than 20%, n = 51	ADAMTS13 activity greater than 20%, n = 52	*P*
Creatinine, mg/dL	1.81 ± 1.70	0.95 ± 0.76	< .01*
AST, IU/L	106 ± 128	182 ± 290	NS
ALT, IU/L	72 ± 109	122 ± 160	NS
Bilirubin, mg/dL	2.70 ± 3.13	2.20 ± 2.53	NS
Albumin, g/dL	2.3 ± 0.4	2.9 ± 0.7	< .05*
CRP, mg/dL	13.50 ± 10.51	6.90 ± 8.61	< .01*
Organ injury, no. (%)			
Renal injury	21 (41.2)	8 (15.4)	< .05†
Liver injury	40 (78.4)	38 (73.1)	NS

Values for all categories except organ injury are mean ± SD. Renal injury: serum creatinine greater than 1.2 mg/dL.

Liver injury: elevation of bilirubin (> 2.0 mg/dL), AST (> 40 IU/L), or ALT (> 40 IU/L).

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

To convert bilirubin from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 17.1.

To convert albumin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert CRP from milligrams per deciliter to milligrams per liter, multiply milligrams per deciliter by 10.

NS indicates not significant.

*Statistically significant (Welch *t* test).

†Statistically significant (Fisher exact probability test).

Analysis of VWF multimers in patients with severe secondary ADAMTS13 deficiency

Additionally, unusually large VWF multimers were detected in the plasma of patients with severe secondary ADAMTS13 deficiency (ADAMTS13 activity less than 20%), as shown in Figure 6. Serum creatinine levels in patients in whom unusually large VWF multimers and severe ADAMTS13 deficiency were detected were significantly higher than in patients in whom the unusually large VWF multimers were absent (Table 4). There was no significant difference in ADAMTS13 activity (Table 4) and ADAMTS13-specific activity (activity-antigen ratio) between these patient groups (not shown).

There was a significant difference in CRP levels between the ADAMTS13 activity less than 20% group and the ADMATS13 activity greater than 20% group, but their platelet counts were not significantly different (not shown), indicating that the decrease in ADAMTS13 may be related to inflammatory responses. These results are consistent with the data showing a negative correlation between the activity and antigen levels of ADAMTS13 and the plasma levels of granulocyte elastase digests of fibrin (E-XDP).

Discussion

ADAMTS13 has been shown to play an important role in VWF processing.[1-14,22,23] As shown previously, ADAMTS13 may cleave the unusually large multimers of VWF on the endothelial cell surface, preventing entrance of such unusually large multimers into the circulation.[8,24] Without this processing of VWF multimers, the unusually large multimers of VWF secreted from endothelial cells would enter the circulation and initiate platelet thrombus formation, which in turn would cause the development of TMA.[8,24] Patients with primary ADAMTS13 deficiency caused by defects in the *ADAMTS13* gene or with autoantibodies against ADAMTS13 have been shown to develop TTP, suggesting the important physiologic role of ADAMTS13-catalyzed cleavage of these

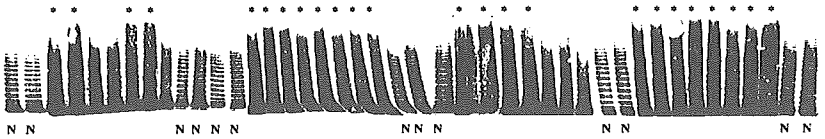

Figure 6. Analysis of VWF multimers of patients with sepsis-induced DIC. VWF multimers in the plasma of patients with sepsis-induced DIC with ADAMTS13 activity levels lower than 20% were analyzed by SDS-agarose gel electrophoresis, as described in "Patients, materials, and methods." VWF multimer patterns of patients and healthy subjects (N) were analyzed simultaneously. *Representative unusually large multimers of VWF found in the plasma of patients with ADAMTS13 activity levels lower than 20%.

unusually large VWF multimers in humans. TTP is a fatal thrombotic microangiopathic disease if patients are not treated appropriately, but the incidence of TTP is low.[8,22] While searching for the role of ADAMTS13 in common thromboembolic diseases, we found severe secondary ADAMTS13 deficiency in patients with sepsis-induced DIC and showed its clinical correlation to the development of renal failure in this study.

DIC is associated with a variety of disease states such as sepsis, advanced malignancy, severe tissue damage, and pregnancy-related complications. Sepsis may be the most common pathogenic disease that leads to the development of DIC, and the endotoxemia and high cytokine levels in the circulation are thought to induce tissue factor expression that in turn initiates fibrin thrombus formation in the circulation. Microthrombi formed in the circulation cause ischemia of and damage to a variety of organs. Lines of evidence have suggested that proteases released from white blood cells may also be involved in the development of organ injuries. This study showed that patients with sepsis-induced DIC frequently exhibited decreased antigen and activity levels of ADAMTS13 and that severe ADAMTS13 deficiency was found in these patients at high incidence. Many patients in this study had undergone transfusion with ADAMTS13-containing blood products, such as fresh frozen plasma and platelet concentrates, soon before blood sample collection for the determination of ADAMTS13 levels, suggesting that the levels of ADAMTS13 in the plasma samples of these patients might not reflect the severity of ADAMTS13 deficiency before blood transfusion. Thus, severe secondary ADAMTS13 deficiency in sepsis-induced DIC might be more common. Clinical manifestations and laboratory data of these patients with sepsis and secondary severe ADAMTS13 deficiency were nearly indistinguishable from those of patients with TTP, though the former had evidence of infection (Table 2), indicating that there exists a subset of patients who have secondary severe ADAMTS13 deficiency caused by sepsis and in whom the disease course is clinically similar to that of TTP. In addition, they might also have the same ADAMTS13 deficiency pathophysiology for the development of TMA seen in patients with idiopathic TTP.

Organ failure might be caused by tissue factor–dependent fibrin thrombus formation and platelet aggregation because of severe ADAMTS13 deficiency in the patients with sepsis-induced DIC with ADAMTS13 activity levels lower than 20%. This notion was supported by the correlation between severe secondary ADAMTS13 deficiency and renal failure in patients with sepsis-induced DIC with ADAMTS13 activity levels lower than 20%. We could not find any significant difference in the ADAMTS13-specific activity levels between these 2 groups (not shown). One possibility is that small molecular forms of ADAMTS13 could be lost in urine because of renal injuries. However, we could not determine whether this was the case because no urine samples were available for study.

Table 4. Correlation between presence of unusually large multimers of VWF and serum creatinine levels of patients with sepsis-induced DIC and ADAMTS13 activity levels lower than 20%

	Presence, n = 26	Absence, n = 25	*P*
Creatinine, mg/dL	2.39 ± 2.24	1.34 ± 1.35	< .05*
ADAMTS13 activity, %	6.6 ± 6.8	8.9 ± 6.0	NS

Values are mean ± SD.

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

Presence indicates unusually large VWF multimers present in the plasma of patients; absence, unusually large VWF multimers absent in the plasma of patients. NS, not significant.

*Statistically significant (Welch *t* test).

In a previous report by Reife et al,[25] patients with TMA who did not have DIC were analyzed for the correlation between ADAMTS13 activity levels and serum creatinine levels without distinguishing TTP from HUS. They found that creatinine levels in patients with severely decreased ADAMTS13 activity levels were significantly lower than those in patients without severely decreased ADAMTS13 activity levels. These data are contrary to our findings that patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) had significantly higher serum creatinine levels than did patients with the ADAMTS13 activity levels higher than 20%. Given that patients with HUS were not distinguished from patients with TTP in the report by Reife et al,[25] it is possible that the patients without severe ADAMTS13 deficiency in that study included patients with HUS. We studied patients with sepsis-induced DIC, and this difference in patient groups explains the opposing findings. There was no apparent difference between the platelet counts of patients with ADAMTS13 activity levels less than 20% and those of patients with ADAMTS13 activity levels greater than 20%. The combination of underlying DIC and platelet transfusion in these patients may account for the data.

The presence of the unusually large multimers of VWF in the plasma of patients with severe secondary ADAMTS13 deficiency and its correlation with serum creatinine levels supports the notion that severe secondary ADAMTS13 deficiency may correlate with the development of renal failure in sepsis-induced DIC. There was no significant correlation between the unusually large multimers of VWF and ADAMTS13 activity levels, possibly because of technical difficulties in determining the unusually large VWF multimers and the differences in endothelial cell damage among these patients.

Decreased specific activity of ADAMTS13, presumably caused by its cleavage by proteases, was a mechanism for severe secondary ADAMTS13 deficiency in patients with sepsis-induced DIC. Various proteases have been shown to degrade ADAMTS13 in vitro.[21] Thrombin and plasmin are generated in DIC, and these enzymes may cleave ADAMTS13, resulting in the inactivation of ADAMTS13. Our data suggest that granulocyte elastase may be one of the proteases that cleave ADAMTS13, together with thrombin and plasmin, under in vivo pathologic conditions. In this regard, the case report by Galbusera et al[26] of chronically relapsing

TTP—which showed that α1-antitrypsin (the physiologic granulocyte elastase inhibitor) therapy was effective at preventing the appearance of unusually large VWF multimers in the circulation but not at preventing TTP relapse—was interesting and suggested the link between granulocyte elastase and cleavage of ADAMTS13. Correlation between ADAMTS13 activity and antigen levels and E-XDP levels, not only in patients with TTP but also in patients with pathogenic *E coli* infection–related HUS, would be a further study to investigate the role of granulocyte elastase in TMA development. Specific inhibitors of these proteases are present at high concentrations in blood, indicating that cleavage of ADAMTS13 by these proteases may depend on the kinetic balance between ADAMTS13, the proteases, and their inhibitors. Thus, cleavage of ADAMTS13 by these proteases may not proceed completely in vivo. It is possible that other proteases could also digest ADAMTS13 in the disease state. This possibility should be investigated in a future study.

Because serum albumin levels decreased in most patients, liver injuries associated with the underlying disease might be an additional mechanism for decreasing ADAMTS13 antigen levels given that this enzyme is synthesized in the liver. Mutations or polymorphisms of the *ADAMTS13* gene are another possible cause of a decrease or an increase of ADAMTS13-specific activity. These possibilities should also be explored in future studies.

In conclusion, the precise analysis of ADAMTS13 antigen and activity levels in disease states offers insight into the roles of ADAMTS13 in thromboembolic diseases. Severe ADAMTS13 deficiency takes place secondarily in disease states such as sepsis-induced DIC, and it may not be specific for idiopathic TTP and may not have a solo diagnostic value for idiopathic TTP. Although the mechanisms of severe ADAMTS13 deficiency in sepsis are different from those of idiopathic TTP, the clinical features of patients with sepsis-induced DIC and severe ADAMTS13 deficiency are similar to those of patients with idiopathic TTP. Sepsis may have the same pathophysiology of severe ADAMTS13 deficiency for TMA development as idiopathic TTP, raising the possibility of novel supportive therapies for patients with sepsis and severe ADAMTS13 deficiency, such as ADAMTS13 supplementation, α1-antitrypsin administration, and use of synthetic granulocyte elastase inhibitors. Given that severe secondary ADAMTS13 deficiency might correlate with the development of organ injury in patients with sepsis-induced DIC, determining the ADAMTS13 levels of patients in severe condition at the time of hospital admission would provide better understanding of the extent of disease. Current analyses of ADAMTS13 levels in disease states are retrospective; thus, prospective study is needed for the timely execution of ADAMTS13 supplementation for patients not only with TTP but also with secondary ADAMTS13 deficiency.

References

1. Gerritsen HE, Robles R, Lämmle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. Blood. 2001;98: 1654-1661.
2. Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of metalloprotease family. Blood. 2001;98:1662-1666.
3. Levy GG, Nicholas WC, Lian EC, et al. Mutation in a member of ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488-494.
4. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS-13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem. 2001;276: 41059-41063.
5. Soejima K, Mimura N, Hirashima M, et al. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? J Biochem (Tokyo). 2001;130:475-480.
6. Moake JL Thrombotic microangiopathies. N Engl J Med. 2002;347:589-600.
7. Furlan M, Robles R, Lamie B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood. 1996;87:4223-4234.
8. Furlan M, Robles R, Galbusera M, et al. Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic uremic syndrome. N Engl J Med. 1998;339:1578-1584.
9. Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. N Engl J Med. 1998; 339:1585-1594.
10. Loof AH, van Vliet HH, Kappers-Klunne MC. Low activity of von Willebrand factor-cleaving protease is not restricted to patients suffering from thrombotic thrombocytopenic purpura. Br J Haematol. 2001;112:1087-1088.
11. Veyradier A, Brivet F, Wolf M, et al. Total deficiency of specific von Willebrand factor-cleaving protease and recovery following plasma therapy in one patient with hemolytic-uremic syndrome. Hematol J. 2001;2:352-354.
12. Remuzzi G, Galbusera M, Noris M, et al. von Willebrand factor cleaving protease (ADAMTS-13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. Blood. 2002;100:778-785.
13. Studt JD, Bohm M, Budde U, Girma JP, Varadi K, Lämmle B. Measurement of von Willebrand factor-cleaving protease (ADAMTS-13) activity in plasma: a multicenter comparison of different assay. J Thromb Haemost. 2003;1:1882-1887
14. Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. Proc Natl Acad Sci U S A. 2002;99:11902-11907.
15. Gando S, Kameue T, Morimoto Y, Matsuda N, Hayakawa M, Kemmotsu O. Tissue factor production not balanced by tissue factor pathway inhibitor in sepsis promotes poor prognosis. Crit Care Med. 2002;30:1729-1734
16. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: Definitions for sepsis and multiple organ failure and guidelines for the use of innovative therapies in sepsis. Crit Care Med. 1992;20:864-874.
17. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975;256:495-497.
18. Soejima K, Matsumoto M, Kokame K, et al. ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. Blood. 2003;102:3232-3237.
19. Ono T, Sogabe M, Ogura M, Furusaki F. Automated latex photometric immunoassay for total plasminogen activator inhibitor-1 in plasma. Clin Chem. 2003;49:987-989.
20. Kohno I, Inuzuka K, Itoh Y, et al. A monoclonal antibody specific to the granulocyte-derived elastase-fragment D species of human fibrinogen and fibrin: its application to the measurement of granulocyte-derived elastase digests in plasma. Blood. 2000;95:1721-1728.
21. Crawley JT, Lam JK, Rance JB, Mollica LR, O'Donnell JS, Lane DA. Proteolytic inactivation of ADAMTS13 by thrombin and plasmin. Blood. 2005;105:1085-1093.
22. Bianchi V, Robles R, Alberio L, Furlan M, Lammle B. von Willebrand factor cleaving protease (ADAMTS-13): in thrombocytopenic disorders: a severely deficient activity is specific for thrombotic thrombocytopenic purpura. Blood. 2002; 100:710-713.
23. Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E. Change in health and disease of the metalloprotease that cleaves von Willebrand factor. Blood. 2001;98:2730-2735.
24. Dong J, Moake JL, Nolasco L, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. Blood. 2002;100: 4033-4039.
25. Raife T, Atkinson B, Montgomery R, Vesely S, Friedman K. Severe deficiency of VWF-cleaving protease (ADAMTS13) activity defines a distinct population of thrombotic microangiopathy patients. Transfusion. 2004;44:146-150.
26. Galbusera M, Ruggenenti P, Noris M, et al. α1-Antitrypsin therapy in a case of thrombotic thrombocytopenic purpura. Lancet. 1995;345:224-225.

ELSEVIER

Cardiovascular Research 66 (2005) 276–285

Cardiovascular Research

www.elsevier.com/locate/cardiores

Review

Aging and plasminogen activator inhibitor-1 (PAI-1) regulation: implication in the pathogenesis of thrombotic disorders in the elderly

Koji Yamamoto[a,*], Kyosuke Takeshita[b], Tetsuhito Kojima[c], Junki Takamatsu[a], Hidehiko Saito[d]

[a]*Department of Transfusion Medicine, Nagoya University Hospital, 65 Tsurumai, Showa, Nagoya 466-8550, Japan*
[b]*Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[c]*Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan*
[d]*Nagoya Medical Center, Nagoya, Japan*

Received 27 August 2004; received in revised form 19 October 2004; accepted 10 November 2004
Available online 8 December 2004
Time for primary review 27 days

Abstract

Thrombotic cardiovascular diseases increase in incidence in the elderly, a tendency dependent on the age-related changes in vascular and hemostatic systems that include platelets, coagulation, and fibrinolytic factors as well as in the endothelium. The hypercoagulability of and advanced sclerotic changes in the vascular wall may contribute to the increased incidence of thrombosis in the elderly. One of the important key genes for aging-associated thrombosis is plasminogen activator inhibitor-1 (PAI-1), a principal inhibitor of fibrinolysis. The expression of PAI-1 is not only elevated in the elderly but also significantly induced in a variety of pathologies associated with the process of aging. These conditions include obesity, insulin resistance, emotional stress, immune responses, and vascular sclerosis/remodeling. Several cytokines and hormones, including tumor necrosis factor-α, transforming growth factor-β, angiotensin II, and insulin, positively regulate the gene expression of PAI-1. The recent epidemic in obesity with aging in the industrialized society may heighten the risk for thrombotic cardiovascular disease because adipose tissue is a primary source of PAI-1 and cytokines. Emotional or psychosocial stress and inflammation also cause the elevated expression of PAI-1 in an age-specific pattern. Thus, PAI-1 could play a key role in the progression of cardiovascular aging by promoting thrombosis and vascular (athero)sclerosis. Further studies on the genetic mechanism of aging-associated PAI-1 induction will be necessary to define the basis for cardiovascular aging in relation to thrombosis.

Keywords: PAI-1; Obesity; Stress; Immune response; Vascular remodeling

1. Introduction

The incidence of thrombotic cardiovascular disease increases with age [1], and recent studies have begun to address the important clinical problem of "aging and thrombosis" [2]. Age-related changes may occur in the vascular and hemostatic systems, which include platelets, coagulation, and fibrinolytic factors as well as in the endothelium. Aging-associated sclerotic changes in the vascular wall may also contribute to the increased incidence of thrombosis in the elderly [3]. The hypercoagulability of the blood in the elderly may be yet another cause of the increased thrombotic tendency. For example, platelet activity is enhanced with advancing age, and aging is associated with increased plasma levels of several blood coagulation factors (e.g., factor VII, factor VIII, and fibrinogen) [4], all of which have been shown to be risk factors for thrombotic diseases [5]. On the other hand, a proportional increase in natural anticoagulant factors (e.g., protein C, protein S, antithrombin, tissue factor pathway inhibitor, etc.) has not been observed in the elderly [6]. The fibrinolytic system is impaired by aging since a progressive prolongation of the euglobulin lysis time [7] and an increase in plasminogen activator inhibitor-1 (PAI-1), a principal

* Corresponding author. Tel.: +81 52 744 2576; fax: +81 52 744 2610.
E-mail address: kojiy@med.nagoya-u.ac.jp (K. Yamamoto).

0008-6363/$ - see front matter © 2004 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2004.11.013

regulator of fibrinolysis [8], have been observed with aging [9]. Thus, the inappropriate expression of procoagulant/antifibrinolytic genes may underlie the occurrence of thrombotic events, which are frequently observed in the elderly. However, the molecular link between aging and prothrombotic states due to aberrant expressions of procoagulant/antifibrinolytic genes remains to be elucidated. One aim of this review is to describe the pathological significance of PAI-1 in cardiovascular aging in relation to thrombosis based upon clinical observations and animal studies.

2. PAI-1 and its regulation in various clinical states associated with aging

PAI-1 is a rapid and specific inhibitor of both tissue-type and urokinase-type plasminogen activators (t-PA and u-PA) and may be the primary regulator of plasminogen activation in vivo [8]. The synthesis of PAI-1 is increased in activated or injured endothelial cells and smooth muscle cells, and abundant PAI-1 is also secreted by activated platelets. The increased expression of this potent inhibitor in vivo will suppress the normal fibrinolytic system and create a prothrombotic state, resulting in pathological fibrin deposition followed by tissue damage. Increased expression of PAI-1 in vivo is related to the development of tissue pathologies [10] such as thrombosis, fibrosis, and cardiovascular disease [11]. Factors inducing PAI-1 expression in vitro and pathologies associated with elevated PAI-1 in vivo are listed in Table 1.

2.1. Myocardial infarction

A rise in the circulating level of PAI-1 has been shown to precede the occurrence of myocardial infarction [12]. Survivors of myocardial infarction had impaired fibrinolytic activity due to elevated levels of plasma PAI-1 [13], which is also associated with early recurrence of myocardial infarction [14]. Acute increases in plasma PAI-1 levels in patients with acute ST-elevated myocardial infarction are strongly associated with the risk of mortality during a 1-month period [15]. Thus, PAI-1 seems to be a risk factor for the development and recurrence of thrombotic cardiovascular diseases. It is also known that the renin–angiotensin system is activated after acute myocardial infarction [16]. A strong relationship has been shown between the activation of the renin–angiotensin system and plasma PAI-1 [17], and it is known that angiotensin II can induce the expression of PAI-1 [18]. The plasma level of another fibrinolytic inhibitor, thrombin-activatable fibrinolysis inhibitor (TAFI), is also associated with increased risk for cardiovascular diseases [19,20]. The activity of TAFI in young patients with myocardial infarction was found to be significantly higher and has been correlated positively with the PAI-1 level [21], suggesting that a hypofibrinolytic state largely contributes to the occurrence of cardiovascular events.

Table 1
Stimulating factors of PAI-1 synthesis and clinical conditions associated with increased PAI-1 expression

Factors inducing PAI-1 synthesis	Clinical conditions with increased PAI-1
Endotoxin	Sepsis
Thrombin	Coronary heart disease
TNF-α	Atherosclerosis
TGF-β	SLE/lupus nephritis
Interleukin-1	Antiphospholipid syndrome
Insulin	Obesity
Dexamethasone	Insulin resistance
PDGF	Lung fibrosis
Basic FGF	Hyperoxic lung injury
Lipoprotein (a)	Preeclampsia
Angiotensin II	Malignancy/tumor metastasis

PDGF—platelet derived growth factor; FGF—fibroblast growth factor; SLE—systemic lupus erythematosus.

2.2. Obesity and insulin resistance

Clinically, thrombotic cardiovascular diseases occurring in aged subjects are often associated with obesity. Obesity is an independent risk factor for the development of thrombotic cardiovascular disease [22]. In a large community-based sample, an increased body-mass index has been associated with increased risk of heart failure [23]. The increased incidence of cardiovascular disease may be associated with impaired fibrinolysis, which has been shown to be present in obese patients [24]. For example, increased plasma PAI-1 levels have been correlated with the amount of visceral fat in obese humans [25], and PAI-1 is commonly and predictably elevated in individuals with insulin resistance and type II diabetes [26]. Vascular dysfunction caused by insulin resistance is associated with the activation of the renin–angiotensin system [27]. Taken together, obesity, insulin resistance, and hypertension are closely related in terms of PAI-1 induction, resulting in the development of thrombotic cardiovascular disease. In this context, we have speculated on the potential benefit of therapies that might prevent an acute increase in plasma PAI-1. These potentially include angiotensin-converting enzyme inhibitors [28], insulin-synthesizing [29] or-sensitizing agents [30], and other agents that improve endothelial function and nitric oxide production systematically.

2.3. Atherosclerosis

By limiting extracellular proteolysis in developing atherosclerotic lesions, PAI-1 may play a significant role not only in the organization of mural thrombi within the plaque but also in the neointimal proliferation of smooth muscle cells and in the neovascularization of the plaque. High plasma levels of PAI-1 may be associated with the development of atherosclerosis. Investigations of PAI-1 expression in the arteries of atherosclerotic subjects have

revealed significantly increased levels of PAI-1 mRNA in severely atherosclerotic vessels, including the abdominal aorta, iliac artery, and femoral artery, as compared with those in normal or mildly affected arteries [31]. In situ hybridization analysis revealed an abundance of cells (e.g., endothelial cells, smooth muscle cells, and macrophages) positive for PAI-1 mRNA within the thickened intima of atherosclerotic arteries, mainly around the base of the plaque [31,32]. Fibrin, which is a consistent component of atherosclerotic plaques, may contribute to plaque growth through the stimulation of smooth muscle cell proliferation [33,34] and through the binding and accumulating of low-density lipoprotein [35]. Intravascular or mural thrombosis is a frequent histological feature of atherosclerotic lesions and appears to play a role in the intimal thickening and fibrosis characteristic of advanced lesions. Thus, localized alterations in fibrinolytic activity due to the increased expression of PAI-1 in blood vessels may contribute to the progression of atherosclerotic process by promoting fibrin deposition and extracellular matrix accumulation in the lesions [36].

2.4. Stress

Hypercoagulability and thrombotic diseases appear to be induced also by mental [37] and psychosocial stress [38]. Because aged subjects may have lower tolerance to stress, they are susceptible to thrombosis caused by a variety of stress factors [39]. Chronic stress, defined as feelings of fatigue, lack of energy, increased irritability, and demoralization, has also been associated with elevated plasma PAI-1 antigen in middle-aged men [40]. The stress-mediated activation of the sympathetic nervous system, whose activity is heightened in older subjects [41], may contribute to the induction of PAI-1 [42]. Oxidative stress, one of the characteristics of diabetes, boosts PAI-1 expression by activating the PAI-1 promoter through an AP-1 response element [43]. Thus, the stress-induced PAI-1 may be responsible for the onset of thrombotic disease associated with a variety of stress factors, especially in the elderly.

2.5. Endotoxemia

PAI-1 is an acute-phase reactant linked to inflammatory and prothrombotic markers because it is induced by a variety of cytokines [e.g., tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-1 and -6], but most strongly by the endotoxin of Gram-negative bacteria [44,45]. Endotoxin (lipopolysaccharide, LPS) profoundly alters the fibrinolytic system [46], frequently leading to prothrombotic states. Recently, PAI-1 has been regarded as a prognostic marker of sepsis caused by Gram-negative bacteria [47], which is often observed in hospitalized elderly patients. Septic patients with high plasma PAI-1 levels have a poor prognosis because of progressive multiple organ failure due to microvascular fibrin deposition and subsequent cell damage [48,49]. After endotoxin administration, elderly individuals are more susceptible to endotoxin-induced effects than the young, showing severe abnormalities in the cardiorespiratory system, such as hypotension, increased heart rate, and increased respiratory rate [50]. Overall, PAI-1 is regarded as a key molecule in the development of septic organ damage because this protein is strongly induced by inflammatory mediators and promotes microvascular and extravascular fibrin deposition.

2.6. Malignancy

A couple of reports have stated that basal plasma PAI-1 levels were found to be significantly elevated in patients with malignant conditions [51], which are sometimes observed in elderly subjects. Deep-vein thrombosis is sometimes observed in patients with malignancy due, not only to the increased activation of coagulation, but also to impaired fibrinolysis. An increasing number of studies demonstrate that high PAI-1 levels indicate a poor prognosis for the survival of patients with a variety of cancers, including breast [52], lung [53], and gastric [54] cancer. PAI-1 may play a critical role in tumor-cell invasion, and the possible mechanism is that PAI-1 blocks the interaction of integrins with vitronectin, thereby loosening the cells from their substratum and promoting cell migration [55].

2.7. Genomics on the PAI-1 up-regulation in relation to thrombosis

The genomics of PAI-1 is relevant to the PAI-1 regulation in association with thrombotic/bleeding phenotype as follows. There have been several reports describing elevated plasma PAI-1 levels in familial or sporadic venous thrombophilia [56]. On the other hand, several individuals have been identified with little or no detectable functional PAI-1 in their plasma due to the mutation in the PAI-1 gene [57], and all have had lifelong bleeding problems [58]. Moreover, disruption of the PAI-1 gene in mice was associated with a mild hyperfibrinolytic state and increased resistance to thrombosis [59]. Transgenic mice overexpressing the human PAI-1 gene developed thrombotic problems in the extremities [60], and an excess of PAI-1 can promote coronary arterial thrombosis in these mice [61]. The coronary thrombi developed in an age-dependent manner in the transgenic mice, and 90% of the mice older than 6 months had spontaneous thrombotic occlusions of the coronary arteries [61].

An association between one of the DNA sequence variations of the human PAI-1 gene, the 4G/5G polymorphism, and plasma PAI-1 levels has been suggested, with the 4G homozygotes having the highest PAI-1 levels and the 5G homozygotes having the lowest [62]. For example, in young myocardial infarction patients, the prevalence of the unfavorable 4G allele was higher than in healthy controls [62]. Furthermore, the 4G/4G genotype has

been shown to be significantly associated with a history of coronary artery disease in patients diagnosed by coronary angiography [63] and also in patients with noninsulin-dependent diabetes mellitus [64,65]. However, it is still controversial whether the 4G/5G polymorphism increases the risk for myocardial infarction and thromboembolism [66].

3. PAI-1 induction in animal models of aging and prothrombotic states

Experimental studies on animals have also demonstrated a link between increased expression of PAI-1 and thrombotic events. In the following, we describe the induction of the PAI-1 gene in a variety of mouse models of aging and prothrombotic states.

3.1. PAI-1 expression in a mouse model of premature aging, "klotho"

A mouse model of premature aging, named the "*klotho* (*kl/kl*) mouse", was generated through the insertional mutation of a transgene disrupting a newly found gene locus named "*klotho*" [67]. The *kl/kl* mouse exhibits a syndrome resembling human aging, including a short life span, growth retardation, osteoporosis, arteriosclerosis, obstructive pulmonary disease, and atrophy of the skin. Higher levels of renal PAI-1 mRNA expression and active PAI-1 antigen in the plasma were found in *kl/kl* mice in comparison with wild-type mice [68], suggesting impaired fibrinolysis in this mouse model of aging. The kidneys of *kl/kl* mice showed severe sclerotic changes, with calcification and spontaneous glomerular fibrin deposition. These observations suggest that the aging-associated induction of PAI-1 contributes to the development of renal sclerotic changes and thrombosis. Interestingly, in the heart of *kl/kl* mice, the cardiomyocytes and the cells in the myxomatous-degenerated mitral valve with calcification also expressed abundant PAI-1 mRNA [68]. The induction of PAI-1 gene expression in cardiomyocytes may contribute to microvascular injury and cardiac muscle degeneration in the hearts of *kl/kl* mice.

3.2. PAI-1 induction in an experimental model of vascular remodeling

One candidate for the paracrine factor involved in vascular remodeling would be the metalloproteinases (MMPs), of which activity is increased in the arteries of aged animals [69]. The plasminnogen activator/plasmin system is an important regulatory system in the onset of cardiac wound healing and arterial remodeling [70] because plasmin can modulate the activity of MMPs by activating proMMPs to MMPs [71]. Age-dependent induction of PAI-1 would enhance the accumulation of ECM components in a variety of tissues, including cardiac and vascular tissues. It has been reported that adenoviral PAI-1 overexpression resulted in the prevention of cardiac rupture after myocardial infarction through the inhibition of local proteolysis [72]. Moreover, PAI-1-deficient mice were found to be resistant to the progression of coronary perivascular fibrous change in a model of long-term nitric oxide (NO) synthase inhibition [73]. Mice deficient in PAI-1 showed less development of cardiac fibrosis after infarction than wild-type mice [74], suggesting that PAI-1 deficiency may prevent the increase of collagen deposition by accelerating matrix degradation. Thus, PAI-1 could regulate the activation of MMPs and has indeed been implicated as an important modulator during the process of cardiac repair and vascular remodeling.

3.3. PAI-1 induction in a mouse model of obesity

High expression levels of PAI-1 mRNA have been detected in murine adipose tissue [75]. This observation suggests that adipose tissue is the primary source of PAI-1 in the obese condition. Adipose-derived PAI-1 expression is dramatically up-regulated and significantly increased as a function of age in genetically obese mice, whose adipocytes express PAI-1 mRNA abundantly [76]. PAI-1 expression in cultured adipocytes has been strongly induced by insulin [76] and glucose [77], suggesting that PAI-1 expression in adipocytes may be strongly associated with insulin resistance [78]. Interestingly, insulin-resistant adipocytes can still respond to insulin stimuli in terms of the induction of the PAI-1 gene [79], suggesting that the expression of PAI-1 is up-regulated by insulin signal independently of insulin sensitivity.

3.4. Stress-induced PAI-1 and thrombosis in association with aging

A dramatic induction of PAI-1 gene has been observed in a mouse restraint-stress model [80], indicating that PAI-1 is a major stress-induced gene. The specific localization of the increased PAI-1 mRNA in epithelial cells, vascular smooth muscle cells, cardiovascular endothelial cells, adrenomedullar chromaffin cells, and neural cells of the para-aortic sympathetic ganglion has been demonstrated in restraint-stressed aged mice [80]. Restraint stress activates the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system, leading to the increased secretion of glucocorticoid hormone and adrenaline, both of which induce PAI-1 expression in vivo [42,81]. The magnitude of PAI-1 mRNA induction due to restraint stress is the highest in the adipose tissue among the tissues examined, and the adipocytes are responsible for this induction [80]. Thus, adipose tissue/adipocytes may be one of the principal sources of PAI-1 expression in response to stress.

More importantly, stress-induced PAI-1 expression has been dramatically enhanced in aged mice [80], indicating an increased ability of aged animals to mount a PAI-1 response to stress. The mRNA induction of a procoagulant gene,

tissue factor (TF), in several tissues due to restraint stress is also higher in aged mice than in young mice [82]. These responses may elevate the procoagulant/antifibrinolytic potential, contributing to the increased incidence of stress-associated thrombotic events in the elderly. Indeed, stress-induced renal glomerular thrombosis is more pronounced in aged mice compared with young mice [80]. This difference in the thrombosis phenotype between young and aged mice may result from a much greater induction of the PAI-1 gene at the systemic and regional levels in aged mice. Thus, an age-related increase in the PAI-1 response to stress may exacerbate vascular injury and subsequent tissue damage as aging progresses.

3.5. Increased microthrombosis with PAI-1 induction in LPS-treated aged animals

Aged rats have shown increased susceptibility to hemorrhaging and intravascular hypercoagulation following endotoxin administration, resulting in a higher mortality of aged rats as compared to young rats [83]. In these studies, a greater increase in PAI-1 activity and a more significant decrease in total PA activity have been demonstrated in the plasma of aged rats treated with endotoxin in comparison with young rats [84]. Interestingly, renal glomerular fibrin deposition and renal PAI-1 gene expression were markedly induced and sustained in LPS-treated aged mice, as compared with young mice [85]. This increased response of the aged mice to LPS in PAI-1 induction, together with the observation that little fibrin was detected in LPS-treated PAI-1 deficient mice, suggests that PAI-1 contributes to an enhanced thrombotic tendency in aged mice suffering from endotoxemia. Thus, aged animals may tend to develop thrombosis due to the high antifibrinolytic potential in endotoxemia and inflammatory processes.

3.6. Enhanced immune response with cytokine induction in aged animals

The expression of CD14, which is a major receptor for LPS on the cell surface triggering a signaling cascade leading to cytokine production [86], has been induced by LPS in a variety of tissues [87]. The expression of CD14 in rat cardiac tissues was found to be more increased in aged animals after LPS treatment, suggesting that innate immune response is augmented with aging [88]. The magnitude of the induction in tissues of CD14 and Toll-like receptor 4 (TLR4), which is identified as another signaling receptor for LPS [89], was found to be greater in LPS-treated aged mice than in young mice [85], suggesting that LPS binding and signaling inside cells is augmented in aged mice. Indeed, higher levels of TNF-α have been detected in the plasma of LPS-treated aged mice in comparison with those of young mice [85], and this response of TNF-α may result in the dramatic induction of PAI-1 in aged mice. Overall, the greater magnitude of the induction of CD14 and TLR4 gene in LPS-treated aged mice may cause a larger increase in PAI-1 expression, leading to enhanced tissue microthrombosis.

Obesity could be considered a low-grade inflammatory state [90]. Several observations indicate that interleukin-6 and TNF-α are elevated in obesity [91], the latter contributing to the insulin-resistant state [92]. Interleukin-6 probably plays an important pathogenic role in a variety of disorders associated with chronic stress and physiological aging [93], such as the induction of PAI-1 [94]. Obese mice treated with neutralizing antibodies to TNF-α not only acquire increased insulin sensitivity but also significantly reduced levels of plasma PAI-1 antigen and adipose-tissue PAI-1 and TGF-β mRNAs [95]. These observations provide direct evidence that TNF-α is a common link between obesity, insulin resistance/hyperinsulinemia, PAI-1, and TGF-β, the last of which is also elevated in obese mice [96]. This establishes a central role for TNF-α in a number of the metabolic disorders associated with obesity. Similar striking elevation of TNF-α was observed in restraint-stressed aged mice [80], suggesting that the induction of cytokines in response to stress is augmented in aged individuals.

4. Procoagulant proteins/molecular markers and platelet function in the elderly

The levels of fibrinogen and factor VIII, both of which are acute-phase reactants, are significantly increased in the elderly [97]. Elevated levels of fibrinogen and factor VIII have been correlated with increased risk of venous thrombosis and cardiovascular events [98,99]. In contrast, factor VII is not an acute-phase reactant and has been identified as an independent risk factor for cardiovascular events [100]. Importantly, factor VIIa is also increased in centenarians [101], suggesting that the coagulation response, initiated by the binding of factor VIIa to TF, is accelerated in the elderly.

Molecular markers of thrombin generation also increase with age [102]. For example, elevated levels of the prothrombin fragment 1+2 (F1+2) have been observed in the elderly, suggesting the presence of excessive plasma factor Xa activity [103]. Other molecules of prothrombotic markers (e.g., fibrinopeptide A and B, factor X-activation peptide, factor IX-activation peptide, and the thrombin–antithrombin complex) have been observed to increase with age [101]. Centenarians have been found to have higher levels of the plasmin–antiplasmin complex and D-dimer compared with younger controls, suggesting a hypercoagulable state with reactive hyperfibrinolysis [101].

Although the proximate cause of elevated coagulation factor levels with aging may be multifactorial, recent studies have demonstrated that certain genomic elements regulate the age dependency of expression. Two genetic elements, AE5′ and AE3′, which contribute to the age-related increase

in factor IX levels, have been discovered in the human factor IX gene [104]. AE5′, which is present in the 5′ untranslated region and a consensus motif for the transcriptional factor, is necessary for the liver-specific expression of the human factor IX gene and for its stable transcription as the individual ages. AE3′, which is an element in the 3′ untranslated region, would increase human factor IX mRNA stability with age. The elements that control age-related gene expression were also discovered in the gene of anticoagulant protein C [105]. However, in general, it appears that the elevation in the anticoagulant proteins levels with aging does not keep pace with that of coagulant protein levels, thus contributing to a prothrombotic state in the elderly [106].

Platelet function is a critical determinant of the propensity to thrombosis, because activated platelets greatly accelerate thrombin generation. Markers of platelet activation, β-thromboglobulin and platelet factor 4, are significantly elevated with age [107]. Platelets from elderly patients may be less susceptible to inhibition by prostacyclin because the density of both high- and low-affinity receptors for prostacyclin decreases with aging [108]. The increase platelet activity with aging is correlated with a larger content of platelet phospholipids, suggesting an age-related increase in platelet transmembrane signaling or second messenger accumulation [109]. Von Willebrand factor, which enhances platelet interaction with the damaged endothelium or subendothelium and which is associated with atherosclerosis, also increases with age [110].

5. Alterations in the vascular wall with aging

Structural changes in the vascular wall at the level of the extracellular matrix, vascular smooth muscle, and endothelium could contribute to the increased risk for thrombosis in the elderly. Advanced age is accompanied by stiffness and dilation of the arteries due to the degeneration of elastic fibers and the increase in collagen content [111]. Gene polymorphisms of elastin and angiotensin II type-I receptor may predispose the elderly to a highly significant age-dependent stiffening and loss of vessel distensibility [112,113]. Aging is also associated with reduced endothelium-dependent dilation [114]. The aged blood vessels express less endothelial nitric oxide (NO) synthase [115], resulting in less NO production [116]. Decreased NO production may contribute to increased platelet activation and arterial thrombosis [117] as well as enhanced atherogenesis [118]. Also, the angiotensin II pathways may play a role in age-related endothelial dysfunction. The expression of angiotensin II is increased in the arterial intima with advancing age [119], and the cardiac expression of receptors for angiotensin II is significantly increased [120]. These observations suggest that age-associated arterial remodeling and the development of atherosclerosis are partially mediated by the increased angiotensin II signaling.

6. Summary

Hypercoagulability and advanced vascular sclerotic changes may contribute to the increased incidence of thrombosis in the elderly. One of the important key genes for the age-associated prothrombotic state is PAI-1 (Fig. 1). The expression of PAI-1 is not only elevated in the elderly but also significantly induced in a variety of pathologies associated with the process of aging. These conditions include obesity, insulin resistance, psychosocial stress, immune responses, and vascular sclerosis/remodeling, all of which accompany aging. Indeed, the expression level of

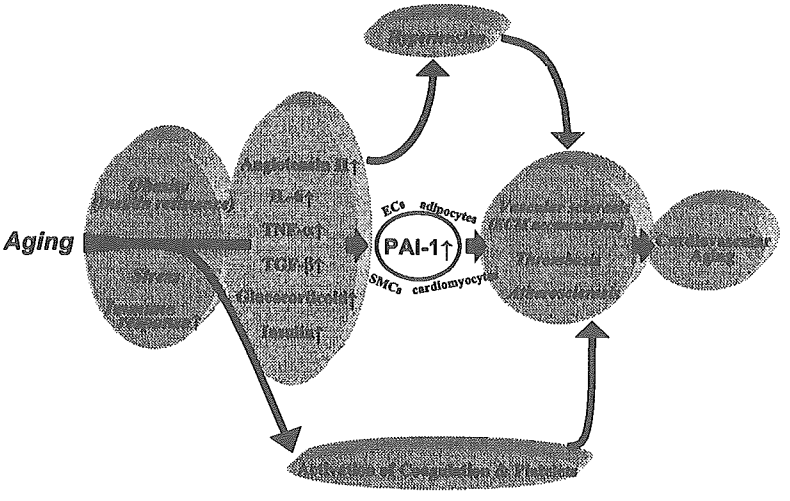

Fig. 1. A key role of PAI-1 in cardiovascular aging. A variety of pathologies associated with aging process cause the PAI-1 induction. This response is enhanced by specific function of several cytokines and hormones. PAI-1 could play a key role in the progression of cardiovascular aging by promoting thrombosis and vascular (athero)sclerosis. ECs—endothelial cells; SMCs—smooth muscle cells.

PAI-1 has been regarded as an important marker for cardiovascular risk. Several cytokines and hormones, including TNF-α, TGF-β, angiotensin II, and insulin, positively regulate the gene expression of PAI-1. These components are primarily synthesized or affected by adipocytes/adipose tissue, which is highlighted because of its relevance to the increased risk for atherosclerosis and cardiovascular events. Thus, PAI-1 could play a key role in the progression of cardiovascular aging and must be considered the most crucial gene for thrombosis and vascular (athero)sclerosis in current developed societies, where the elderly, the obese, and individuals exposed to stress are increasing in number. Further studies on the mechanism of age-regulated expression of PAI-1 are necessary in order to define the basis for cardiovascular aging in relation to thrombosis. It is also important for future clinical research to establish the most promising strategies for controlling PAI-1 expression so that cardiovascular diseases associated with aging can be prevented.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Science, Sports and Culture, from the Ministry of Health and Welfare, Japan.

References

[1] Tracy RP, Bovill EG. Thrombosis and cardiovascular risk in the elderly. Arch Pathol Lab Med 1992;116:1307–12.
[2] Wilkerson WR, Sane DC. Aging and thrombosis. Semin Thromb Hemost 2002;28:555–67.
[3] Kiechl S, Willeit J. The natural course of atherosclerosis: Part II. Vascular remodeling. Arterioscler Thromb Vasc Biol 1999;19: 1491–8.
[4] Balleisen L, Bailey J, Epping PH, Schulte H, van de Loo J. Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population: I. Baseline data on the relation to age, gender, body-weight, smoking, alcohol, pill-using, and menopause. Thromb Haemost 1985;54:475–9.
[5] Koster T, Rosendaal FR, Reitsma PH, van der Velden PA, Briet E, Vandenbroucke JP. Factor VII and fibrinogen levels as risk factors for venous thrombosis. A case-control study of plasma levels and DNA polymorphisms—The Leiden Thrombophilia Study (LETS). Thromb Haemost 1994;71:719–22.
[6] Sagripanti A, Carpi A. Natural anticoagulants, aging, and thromboembolism. Exp Gerontol 1998;33:891–6.
[7] Abbate R, Prisco D, Rostagno C, Boddi M, Gensini GF. Age-related changes in the hemostatic system. Int J Clin Lab Res 1993; 23:1–3.
[8] Loskutoff DJ, Sawdey M, Mimuro J. Type 1 plasminogen activator inhibitor. In: Coller B, editor. Progress in hemostasis and thrombosis. Philadelphia: WB Saunders; 1988. p. 87–115.
[9] Hashimoto Y, Kobayashi A, Yamazaki N, Sugawara Y, Takada Y, Takada A. Relationship between age and plasma t-PA, PA-inhibitor, and PA activity. Thromb Res 1987;46:625–33.
[10] Yamamoto K, Saito H. A pathological role of increased expression of plasminogen activator inhibitor-1 in human or animal disorders. Int J Hematol 1998;68:371–85.
[11] Kohler HP, Grant P. Plasminogen activator inhibitor type 1 and coronary artery disease. N Engl J Med 2000;342:1792–801.
[12] Juhan-Vague I, Pyke SD, Alessi MC, Jespersen J, Haverkate F, Thompson SG. Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. ECAT study group. European concerted action on thrombosis and disabilities. Circulation 1996;94:2057–63.
[13] Hamsten A, Wiman B, de Faire U, Blomback M. Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. N Engl J Med 1985;313:1557–63.
[14] Hamsten A, de Faire U, Walldius G. Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987;2:3–9.
[15] Collet JP, Montalescot G, Vicaut E, Ankri A, Walylo F, Lesty C, et al. Acute release of plasminogen activator inhibitor-1 in ST-segment elevation myocardial infarction predicts mortality. Circulation 2003;108:391–4.
[16] Rouleau JL, Moye LA, de Champlain J, Klein M, Bichet D, Packer M, et al. Activation of neurohumoral systems following acute myocardial infarction. Am J Cardiol 1991;68:80D–6D.
[17] Brown NJ, Agirbasli MA, Williams GH, Litchfield WR, Vaughan DE. Effect of activation and inhibition of the renin-angiotensin system on plasma PAI-1. Hypertension 1998;32:965–71.
[18] Vaughan DE, Lazos SA, Tong K. Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin-angiotensin system and thrombosis. J Clin Invest 1995;95:995–1001.
[19] Juhan-Vague I, Renucci JF, Grimaux M, Morange PE, Gouvernet J, Gourmelin Y, et al. Thrombin-activatable fibrinolysis inhibitor antigen levels and cardiovascular risk factors. Arterioscler Thromb Vasc Biol 2000;20:2156–61.
[20] Santamaria A, Borrell M, Oliver A, Ortin R, Forner R, Coll I, et al. Association of functional thrombin-activatable fibrinolysis inhibitor (TAFI) with conventional cardiovascular risk factors and its correlation with other hemostatic factors in a Spanish population. Am J Hematol 2004;76:348–52.
[21] Zorio E, Castello R, Falco C, Espana F, Osa A, Almenar L, et al. Thrombin-activatable fibrinolysis inhibitor in young patients with myocardial infarction and its relationship with the fibrinolytic function and the protein C system. Br J Haematol 2003;122:958–65.
[22] Larsson B. Obesity, fat distribution and cardiovascular disease. Int J Obes 1991;15:53–7.
[23] Kenchaiah S, Evans JC, Levy D, Wilson PW, Benjamin EJ, Larson MG, et al. Obesity and the risk of heart failure. N Engl J Med 2002;347:305–13.
[24] McGill JB, Schneider DJ, Arfken CL, Lucore CL, Sobel BE. Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. Diabetes 1994;43:104–9.
[25] Shimomura I, Funahashi T, Takahashi M, Maeda K, Kotani K, Nakamura T, et al. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. Nat Med 1996; 2:800–3.
[26] Lyon CJ, Hsueh WA. Effect of plasminogen activator inhibitor-1 in diabetes mellitus and cardiovascular disease. Am J Med 2003;115(Suppl 8A):62S–8S.
[27] Shinozaki K, Ayajiki K, Nishio Y, Sugaya T, Kashiwagi A, Okamura T. Evidence for a causal role of the rennin-angiotensis system in vascular dysfunction associated with insulin resistance. Hypertension 2004;43:255–62.
[28] Vaughan DE, Rouleau JL, Ridker PM, Arnold JM, Menapace FJ, Pfeffer MA. Effects of ramipril on plasma fibrinolytic balance in patients with acute anterior myocardial infarction. Circulation 1997; 96:442–7.
[29] Vague P, Juhan-Vague I, Alessi MC, Badier C, Valadier J. Metformin decreases the high plasminogen activator inhibition capacity, plasma insulin and triglyceride levels in non-diabetic obese subjects. Thromb Haemost 1987;57:326–8.

[30] Zirlik A, Leugers A, Lohrmann J, Ernst S, Sobel BE, Bode C, et al. Direct attenuation of plasminogen activator inhibitor type-1 expression in human adipose tissue by thiazolidinediones. Thromb Haemost 2004;91:674–82.
[31] Schneiderman J, Sawdey MS, Keeton MR, Bordin GM, Bernstein EF, Dilley RB, et al. Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. Proc Natl Acad Sci U S A 1992;89:6998–7002.
[32] Chomiki N, Henry M, Alessi MC, Anfosso F, Juhan-Vague I. Plasminogen activator inhibitor-1 expression in human liver and healthy or atherosclerotic vessel walls. Thromb Haemost 1994; 72:44–53.
[33] Bini A, Fenoglio JJ, Mesa-Tejada R, Kudryk B, Kaplan KK. Identification and distribution of fibrinogen, fibrin and fibrin(ogen) degradation products in atherosclerosis. Arteriosclerosis 1989; 9:109–21.
[34] Naito M, Funaki C, Hayashi T, Yamada K, Asai K, Yoshimine N, et al. Substrate-bound fibrinogen, fibrin and other cell attachment-promoting proteins as a scaffold for cultured vascular smooth muscle cells. Atherosclerosis 1992;96:227–34.
[35] Smith EB, Cochran S. Factors influencing the accumulation in fibrous plaque of lipid derived from low density lipoprotein: Part II. Preferential immobilization of lipoprotein (a). Atherosclerosis 1990;84:173–81.
[36] Eitzman DT, Westrick RJ, Xu Z, Tyson J, Ginsburg D. Plasminogen activator inhibitor-1 deficiency protects against atherosclerosis progression in the mouse carotid artery. Blood 2000;96:4212–5.
[37] Jern C, Eriksson E, Tengborn L, Risberg B, Wadenvik H, Jern S. Changes of plasma coagulation and fibrinolysis in response to mental stress. Thromb Haemost 1989;62:767–71.
[38] Rosengren A, Hawken S, Ôunpuu S, Sliwa K, Zubaid M, Almahmeed WA, et al. Association of psychosocial risk factors with risk of acute myocardial infarction in 11119 cases and 13648 controls from 52 countries (the INTERHEART study): case-control study. Lancet 2004;364:953–62.
[39] Lecomte D, Fornes P, Nicolas G. Stressful events as a trigger of sudden death: a study of 43 medico-legal autopsy cases. Forensic Sci Int 1996;79:1–10.
[40] Raikkonen K, Lassila R, Keltikangas-Jarvinen L, Hautanen A. Association of chronic stress with plasminogen activator inhibitor-1 in healthy middle-aged men. Arterioscler Thromb Vasc Biol 1996;16:363–7.
[41] Supiano MK, Hogikyan RV, Sidani MA, Galecki AT, Krueger JL. Sympathetic nervous system activity and α-adrenergic responsiveness in older hypertensive humans. Am J Physiol 1999; 276:E519–28.
[42] Larsson PT, Wiman B, Olsson G, Angelin B, Hjemdahl P. Influence of metoprolol treatment on sympatho-adrenal activation of fibrinolysis. Thromb Haemost 1990;63:482–7.
[43] Vulin AI, Stanley FM. Oxidative stress activates the plasminogen activator inhibitor type 1 (PAI-1) promoter through an AP-1 response element and cooperates with insulin for additive effects on PAI-1 transcription. J Biol Chem 2004;279:25172–8.
[44] Sawdey MS, Loskutoff DJ. Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-α, and transforming growth factor-β. J Clin Invest 1991;88:1346–53.
[45] Yamamoto K, Loskutoff DJ. Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plasminogen activator. J Clin Invest 1996;97:2440–51.
[46] Suffredini AF, Harpel PC, Parrillo JE. Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects. N Engl J Med 1989;320: 1165–72.
[47] Pralong G, Calandra T, Glauser M-P, Schellekens J, Verhoef J, Bachmann F, et al. Plasminogen activator inhibitor 1: a new prognostic marker in septic shock. Thromb Haemost 1989;61: 459–62.
[48] Mesters RM, Florke N, Ostermann H, Kienast J. Increase of plasminogen activator inhibitor levels predicts outcome of leukocytopenic patients with sepsis. Thromb Haemost 1996;75:902–7.
[49] Green J, Doughty L, Kaplan SS, Sasser H, Carcillo JA. The tissue factor and plasminogen activator inhibitor type-1 response in pediatric sepsis-induced multiple organ failure. Thromb Haemost 2002;87:218–23.
[50] Horan MA, Hendriks HFJ, Brouwer A. Organisms under stress: infectious agents and host defenses. In: Horan MA, Brouwer A, editors. Gerontology. Approaches to biomedical and clinical research. London: Edward Arnold; 1990. p. 105–34.
[51] Newland JR, Haire WD. Elevated plasminogen activator inhibitor levels found in patients with malignant conditions. Am J Clin Pathol 1991;96:602–4.
[52] Grondahl-Hansen J, Christensen IJ, Rosenquist C, Brunner N, Mouridsen HT, Dano K, et al. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 1993;53:2513–21.
[53] Pedersen H, Brunner N, Francis D, Osterlind K, Ronne E, Hansen HH, et al. Prognostic impact of urokinase, urokinase receptor, and type 1 plasminogen activator inhibitor in squamous and large cell lung cancer tissue. Cancer Res 1994;54:4671–5.
[54] Nekarda H, Schmitt M, Ulm K, Wenninger A, Vogelsang H, Becker K, et al. Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer. Cancer Res 1994;54:2900–7.
[55] Deng G, Curriden SA, Hu G, Czekay RP, Loskutoff DJ. Plasminogen activator inhibitor-1 regulates cell adhesion by binding to the somatomedin B domain of vitronectin. J Cell Physiol 2001;189: 23–33.
[56] Engesser L, Brommer EJP, Kluft C, Briet E. Elevated plasminogen activator inhibitor (PAI), a cause of thrombophilia?—A study in 203 patients with familial or sporadic venous thrombophilia. Thromb Haemost 1989;62:673–80.
[57] Fay WP, Shapiro AD, Shih JL, Schleef RR, Ginsburg D. Complete deficiency of plasminogen activator inhibitor type 1 due to a frameshift mutation. N Engl J Med 1992;327:1729–33.
[58] Schleef RR, Higgins DL, Pillemer E, Levitt LJ. Bleeding diathesis due to decreased functional activity of type 1 plasminogen activator inhibitor. J Clin Invest 1989;83:1747–52.
[59] Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, et al. Plasminogen activator inhibitor-1 gene-deficient mice: II. Effects on hemostasis, thrombosis, and thrombolysis. J Clin Invest 1993;92:2756–60.
[60] Erickson LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR. Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. Nature 1990;346:74–6.
[61] Eren M, Painter CA, Atkinson JB, Declerck PJ, Vaughan DE. Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1. Circulation 2002;106:491–6.
[62] Eriksson P, Kallin B, Van'T Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. Proc Natl Acad Sci U S A 1995;92:1851–5.
[63] Ossei-Gerning N, Mansfield MW, Stickland MH, Wilson IJ, Grant PJ. Plasminogen activator inhibitor-1 promoter 4G/5G genotype and plasma levels in relation to a history of myocardial infarction in patients characterized by coronary angiography. Arterioscler Thromb Vasc Biol 1997;17:33–7.
[64] Panahloo A, Mohamed-Ali V, Lane A, Green F, Humphries SE, Yudkin JS. Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene. Diabetes 1995;44:37–42.

[65] Mansfield MW, Stickland MH, Grant PJ. Plasminogen activator inhibitor-1 (PAI-1) promoter polymorphism and coronary artery disease in non-insulin-dependent diabetes. Thromb Haemost 1995; 74:1032–4.
[66] Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Miletich JP. Arterial and venous thrombosis is not associated with the 4G/5G polymorphism in the promoter of the plasminogen activator inhibitor gene in a large cohort of US men. Circulation 1997;95:59–62.
[67] Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 1997;390:45–51.
[68] Takeshita K, Yamamoto K, Ito M, Kondo T, Matsushita T, Hirai M, et al. Increased expression of plasminogen activator inhibitor-1 with fibrin deposition in a murine model of aging, "*klotho*" mouse. Semin Thromb Hemost 2002;28:545–53.
[69] Wang M, Lakatta EG. Altered regulation of matrix metalloproteinase-2 in aortic remodeling during aging. Hypertension 2002; 39:865–73.
[70] Creemers EEJM, Cleutjens J, Smits JFM, Daemen MJAP. Matrix metalloproteinase inhibition after myocardial infarction. A new approach to prevent heart failure? Circ Res 2001;89:201–10.
[71] Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, et al. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet 1997;17:439–44.
[72] Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevent cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med 1999;10:1135–42.
[73] Kaikita K, Fogo AB, Ma L, Schoenhard JA, Brown NJ, Vaughan DE. Plasminogen activator inhibitor-1 deficiency prevents hypertension and vascular fibrosis in response to long-term nitric oxide synthase inhibition. Circulation 2001;104:839–44.
[74] Takeshita K, Hayashi M, Iino S, Kondo T, Inden Y, Iwase M, et al. Increased expression of plasminogen activator inhibitor-1 in cardiomyocytes contributes to cardiac fibrosis after myocardial infarction. Am J Pathol 2004;164:449–56.
[75] Samad F, Yamamoto K, Loskutoff DJ. Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo: induction by tumor necrosis factor-α and lipopolysaccharide. J Clin Invest 1996;97:37–46.
[76] Samad F, Loskutoff DJ. Tissue distribution and regulation of plasminogen activator inhibitor-1 in obese mice. Mol Med 1996;2:568–82.
[77] Gabriely I, Yang XM, Cases JA, Ma XH, Rossetti L, Barzilai N. Hyperglycemia induces PAI-1 gene expression in adipose tissue by activation of the hexosamine biosynthetic pathway. Atherosclerosis 2002;160:115–22.
[78] Potter van Loon BJ, Kluft C, Radder JK, Blankenstein MA, Meinders AE. The cardiovascular risk factor plasminogen activator inhibitor type 1 is related to insulin resistance. Metabolism 1993; 42:945–9.
[79] Samad F, Pandey M, Bell PA, Loskutoff DJ. Insulin continues to induce plasminogen activator inhibitor 1 gene expression in insulin-resistant mice and adipocytes. Mol Med 2000;6:680–92.
[80] Yamamoto K, Takeshita K, Shimokawa T, Yi H, Isobe K, Loskutoff DJ, et al. Plasminogen activator inhibitor-1 is a major stress-regulated gene: implications for stress-induced thrombosis in aged individuals. Proc Natl Acad Sci U S A 2002;99:890–5.
[81] Konkle BA, Schuster SJ, Kelly MD, Harjes K, Hassett DE, Bohrer M, et al. Plasminogen activator inhibitor-1 messenger RNA expression is induced in rat hepatocytes in vivo by dexamethasone. Blood 1992;79:2636–42.
[82] Yamamoto K, Shimokawa T, Yi H, Isobe K, Kojima T, Loskutoff DJ, et al. Aging and obesity augment the stress-induced expression of tissue factor gene in the mouse. Blood 2002;100:4011–8.
[83] Carthew P, Dorman BM, Edwards RE. Increased susceptibility of aged rats to haemorrhage and intravascular hypercoagulation following endotoxin administered in a generalized Shwartzman regime. J Comp Pathol 1991;105:323–30.
[84] Emeis JJ, Brouwer A, Barelds J, Horan MA, Durham SK, Kooistra T. On the fibrinolytic system in aged rats, and its reactivity to endotoxin and cytokines. Thromb Haemost 1992;67:697–701.
[85] Yamamoto K, Shimokawa T, Yi H, Isobe K, Kojima T, Loskutoff DJ, et al. Aging accelerates endotoxin-induced thrombosis: increased responses of plasminogen activator inhibitor-1 and LPS signaling with aging. Am J Pathol 2002;161:1805–14.
[86] Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding proteins. Science 1990;249:1431–3.
[87] Fearns C, Kravchenko VV, Ulevitch RJ, Loskutoff DJ. Murine CD14 gene expression in vivo. Extramyeloid synthesis and regulation by lipopolysaccharide. J Exp Med 1995;181:857–66.
[88] Rosas GO, Zieman SJ, Donabedian M, Vandegaer K, Hare JM. Augmented age-associated innate immune responses contribute to negative inotropic and lusitropic effects of lipopolysaccharide and interferon gamma. J Mol Cell Cardiol 2001;33:1849–59.
[89] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the lps gene product. J Immunol 1999;162:3749–52.
[90] Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 2000;148:209–14.
[91] Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest 1995;95:2111–9.
[92] Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. J Clin Invest 1995;95:2409–15.
[93] Papanicolau DA, Wilder RL, Manolagas SC, Chrousos GP. The pathophysiologic roles of interleukin-6 in human disease. Ann Intern Med 1998;128:127–37.
[94] Mestries J-C, Kruithof EKO, Gascon M-P, Herodin F, Agay D, Ythier A. In vivo modulation of coagulation and fibrinolysis by recombinant glycosylated human interleukin-6 in baboons. Eur Cytokine Netw 1994;5:275–81.
[95] Samad F, Uysal KT, Wiesbrock SM, Pandey M, Hotamisligil GS, Loskutoff DJ. Tumor necrosis factor α is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1. Proc Natl Acad Sci U S A 1999;96:6902–7.
[96] Samad F, Yamamoto K, Pandey M, Loskutoff DJ. Elevated expression of transforming growth factor-β in adipose tissue from obese mice. Mol Med 1997;3:36–47.
[97] Tracy RP, Bovill EG, Fried LP, Heiss G, Lee MH, Polak JF, et al. The distribution of coagulation factors VII and VIII and fibrinogen in adults over 65 years. Results from the Cardiovascular Health Study. Ann Epidemiol 1992;2:509–19.
[98] Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. N Engl J Med 1984;311:501–5.
[99] Koster T, Blann D, Briet E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII and effect of von Willebrand factor on occurrence of deep-vein thrombosis. Lancet 1995;345:152–5.
[100] Junker R, Heinrich J, Schulte H, van de Loo J, Assmann G. Coagulation factor VII and the risk of coronary heart disease in healthy men. Arterioscler Thromb Vasc Biol 1997;17:1539–44.
[101] Mari D, Mannucci PM, Coppola R, Bottasso B, Bauer KA, Rosenberg RD. Hypercoagulability in centenarians: the paradox of successful aging. Blood 1995;85:3144–9.
[102] Bauer KA, Weiss LM, Sparrow D, Vokonas PS, Rosenberg RD. Aging-associated changes in indices of thrombin generation and protein C activation in humans. Normative aging study. J Clin Invest 1987;80:1527–34.

[103] Cushman M, Psaty BM, Macy E, Bovill EG, Cornell ES, Kuller LH, et al. Correlates of thrombin markers in an elderly cohort free of clinical cardiovascular disease. Arterioscler Thromb Vasc Biol 1996;16:1163–9.
[104] Kurachi S, Deyashiki Y, Takeshita J, Kurachi K. Genetic mechanisms of age regulation of human blood coagulation factor IX. Science 1999;285:739–43.
[105] Zhang K, Kurachi S, Kurachi K. Genetic mechanisms of age regulation of protein C and blood coagulation. J Biol Chem 2002; 277:4532–40.
[106] Sakkinen PA, Cushman M, Psaty BM, Kuller LH, Bajaj SP, Sabharwal AK, et al. Correlates of antithrombin, protein C, protein S, and TFPI in a healthy elderly cohort. Thromb Haemost 1998; 80:134–9.
[107] Zahavi J, Jones NAG, Leyton J, Dubiel M, Kakkar VV. Enhanced in vivo "platelet reaction" in old healthy individuals. Thromb Res 1980;17:329–36.
[108] Modesti PA, Fortini A, Abbate R, Gensini GF. Age related changes of platelet prostacyclin receptors in humans. Eur J Clin Invest 1985; 15:204–8.
[109] Bastyr III EJ, kadrofske MM, Vinik AI. Platelet activity and phosphoinositide turnover increase with advancing age. Am J Med 1990;88:601–6.
[110] Conlan MG, Folsom AR, Finch A, Davis CE, Sorlie P, Marcucci G, et al. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. Thromb Heamost 1993; 70:380–5.
[111] Lakatta EG, Mitchell JH, Pomerance A, Rowe GG. Human aging: changes in structure and function. J Am Coll Cardiol 1987;10: 42A–7A.
[112] Hanon O, Luong V, Mourad JJ, Bortolotto LA, Jeunemaitre X, Girerd X. Aging, carotid artery distensibility, and the Ser422Gly elastic gene polymorphism in humans. Hypertension 2001;38: 1185–9.
[113] Lajemi M, Labat C, Gautier S, Lacolley P, Safar M, Asmar R, et al. Angiotensin II type 1 receptor $^{-153}$A/G and ^{1166}A/C gene polymorphisms and increase in aortic stiffness with age in hypertensive subjects. J Hypertens 2001;19:407–13.
[114] Celermajer DS, Sorensen KE, Bull C, Robinson J, Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interactions. J Am Coll Cardiol 1994;24:1468–74.
[115] Chou T-C, Yen M-H, Li C-Y, Ding Y-A. Alterations of nitric oxide synthase expression with aging and hypertension. Hypertension 1998;31:643–8.
[116] Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, et al. Age-related reduction of NO availability and oxidative stress in humans. Hypertension 2001;38:274–9.
[117] Loscalzo J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res 2001;88:756–62.
[118] Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774–7.
[119] Wang M, Takagi G, Asai K, Resuello RG, Natividad FF, Vatner DE, et al. Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. Hypertension 2003;41:1308–16.
[120] Heymes C, Silvestre J-S, Llorens-Cortes C, Chevalier B, Marotte F, Levy BI, et al. Cardiac senescence is associated with enhanced expression of angiotensin II receptor subtypes. Endocrinology 1998;139:2579–87.

Journal of Thrombosis and Haemostasis, 3: 1495–1502

ORIGINAL ARTICLE

Obesity enhances the induction of plasminogen activator inhibitor-1 by restraint stress: a possible mechanism of stress-induced renal fibrin deposition in obese mice

K. YAMAMOTO,* T. KOJIMA,† T. ADACHI,‡ M. HAYASHI,§ T. MATSUSHITA,‡ J. TAKAMATSU,* D. J. LOSKUTOFF¶ and H. SAITO**

**Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan; †Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan; ‡Department of Hematology, Nagoya University Graduate School of Medicine, Nagoya, Japan; §Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan; ¶Department of Cell Biology (VB-3), The Scripps Research Institute, La Jolla, CA, USA; and **Nagoya National Hospital, Nagoya, Japan*

To cite this article: Yamamoto K, Kojima T, Adachi T, Hayashi M, Matsushita T, Takamatsu J, Loskutoff DJ, Saito H. Obesity enhances the induction of plasminogen activator inhibitor-1 by restraint stress: a possible mechanism of stress-induced renal fibrin deposition in obese mice. *J Thromb Haemost* 2005; 3: 1495–1502.

Summary. *Background and objectives:* Cardiovascular/thrombotic diseases are frequently induced by a variety of stressors. Obese patients are susceptible to thrombotic diseases associated with stress, but the underlying mechanism is still unknown. We have begun to investigate the expression of a primary inhibitor of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1), in association with tissue thrombosis, using restraint-stressed obese mice. *Methods and results:* We analyzed the expression of PAI-1 after restraint (immobilization) stress in genetically obese mice in comparison with their lean counterparts. Dramatic increases in PAI-1 antigen in plasma and in tissue extracts were observed in the obese mice exposed to restraint stress. The induction of PAI-1 mRNA by stress in the tissues was also pronounced in the stressed obese mice as compared with the lean mice, especially in the hearts and adipose tissues. *In situ* hybridization analysis revealed that strong signals for PAI-1 mRNA were localized in the adipocytes, cardiovascular endothelial cells, and renal glomerular cells of the stressed obese mice. Histological examination revealed that renal glomerular fibrin deposition was detected only in the obese mice after 2 h of restraint stress. *Conclusions:* Obesity enhances the stress-mediated PAI-1 induction in the blood and tissues. This phenomenon may be associated with the increased risk of stress-induced renal fibrin deposition in obese subjects.

Keywords: adipose, fibrin deposition, obesity, PAI-1, stress.

Correspondence: Dr K. Yamamoto, Department of Transfusion Medicine, Nagoya University Hospital, 65 Tsurumai, Showa, Nagoya 466-8550, Japan.
Tel.: +81 52 744 2576; fax: +81 52 744 2610; e-mail: kojiy@med.nagoya-u.ac.jp

Received 13 December 2004, accepted 4 March 2005

Introduction

Obesity is an independent risk factor for the development of cardiovascular/thrombotic disease [1,2]. The increased incidence of cardiovascular disease may be associated with elevated levels of coagulation factors (e.g. factor VII, fibrinogen) and plasminogen activator inhibitor-1 (PAI-1) in plasma, which have been observed in obese patients [3,4]. PAI-1 is the primary inhibitor of plasminogen activation *in vivo*, and increased PAI-1 in plasma compromises the normal fibrin clearance mechanisms, promoting thrombosis [5], which can take the form of coronary artery disease [6]. In obese humans, increased plasma PAI-1 levels have been correlated with the amount of visceral fat [7], suggesting that adipose tissue is the primary source of PAI-1 in this condition. For example, relatively high levels of PAI-1 mRNA have been detected in the human [8] and murine adipose tissue [9], and adipose-derived PAI-1 expression has been found to be dramatically increased in genetically obese mice [10]. PAI-1 expression in cultured adipocytes is strongly upregulated by glucocorticoids [11], insulin [12], tumor necrosis factor-α [9], and transforming growth factor-β [12], all of which have been found to be elevated in obese subjects [13].

Mental and physical stressors decrease fibrinolytic activity [14,15] and contribute to the occurrence of thrombotic complications. For example, PAI-1 has been strongly induced by acute inflammatory stress [16] and hypoxic stress [17] *in vivo*. Chronic stress, defined as feelings of fatigue, increased irritability, and demoralization, has also been associated with elevated plasma PAI-1 antigen levels in humans [18]. Recently, the presence of psychosocial stressors, including financial stress, stress at the workplace and home, and major life events during the past year, has been associated with increased risk of acute myocardial infarction [19]. We have also reported that PAI-1 expression is dramatically induced by restraint (immobilization) stress, a typical physicopsychological stress [20], with maximal induction in the adipose tissue *in vivo*, a change

contributing to the development of tissue thrombosis [21]. In this context, obese individuals are susceptible to stress-mediated pathological changes, including thrombotic complications [22], possibly because of the stress-induced imbalance of the coagulation and fibrinolytic systems, and thus, obese animals may have lower tolerance to stress insults [23]. Taken together, these observations have led us to hypothesize that obesity may enhance the stress-mediated induction of PAI-1, thus causing thrombosis in obese patients.

In the present study, the effect of obesity on stress-induced PAI-1 expression and subsequent tissue thrombosis was investigated using genetically obese mice. A greater induction of the PAI-1 gene in response to restraint stress was observed in obese mice as compared with their lean counterparts, and renal glomerular fibrin deposition was induced only in the stressed obese mice. These observations suggest a possible mechanism of stress-induced fibrin deposition in the tissue of obese subjects.

Materials and methods

Restraint stress and tissue preparation

Male obese mice (C57BL/6J ob/ob) of 6 weeks old and their lean counterparts (C57BL/6J +/?) were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). The mice were placed into conical centrifuge tubes fitted with multiple punctures so as to allow ventilation. We used 50 mL-tubes (20 mm in diameter) for lean mice and 100 mL-tubes (30 mm in diameter) for obese mice to subject them to the same level of restraint stress. The tubes were placed in horizontal holders and the animals thus maintained for a continuous period of restraint [20]. During this time, the animals were provided with water only. After 2 or 20 h of restraint, the mice ($n = 8$ in each time point) were sacrificed by overdose inhalation anesthesia with methoxyflurane (Pitman-Moore, Mundelein, MD, USA), which did not influence the PAI-1 expression (not shown). This experimental protocol was approved by the Animal Care and Use Committee of Nagoya University. Tissues were rapidly removed by standard dissection techniques, and either minced and immediately frozen in liquid nitrogen for preparation of total RNA or protein extraction. Other portions of tissues were fixed in chilled 4% paraformaldehyde and embedded in paraffin for *in situ* hybridization or for fibrin immunohistochemistry.

Determination of PAI antigen in mouse plasma and tissue extracts

The blood was collected into 20 mM EDTA (final concentration), centrifuged at 3000 *g* for 5 min, and then the plasma was removed and stored at −70 °C. Extraction of tissues was performed as described [24], and the protein concentration of the supernatant was determined by bicinchoninic acid (BCA) assay. The tissue extracts were also stored at −70 °C. PAI-1 antigen in the plasma and in the tissue extracts was determined by employing enzyme-linked immunosorbent assay (ELISA) specific for murine PAI-1, which was established in our laboratory [25]. The results are expressed as nanogram of PAI-1 per milliliter of plasma or picogram of PAI-1 per milligram of tissue.

Quantitative reverse transcription-polymerase chain reaction

We have developed a quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay to determine the concentration of PAI-1 mRNA in murine tissues as described in previous studies [16]. Briefly, total RNA was prepared from unfixed tissues using the Ultraspec^{TM} RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX, USA), and then quantified by measuring absorption at 260 nm. The complementary RNA (cRNA) standard was then *in vitro* transcribed using the Riboprobe Gemini II (Promega, Madison, WI, USA). Thereafter, 1 μg of total tissue RNA and the cRNA standard to be used as a competitor for the target mRNA were combined and reverse transcribed using a Gene Amp RNA PCR kit (Perkin-Elmer/Cetus, Norwalk, CT, USA). Serial twofold dilutions of the RT mixture were amplified using specific primers for PAI-1 or β-actin in the presence of ^{32}P-end-labeled sense primer (5×10^5 cpm). After PCR amplification for 30–35 cycles (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min), 20-μL of aliquots of the PCR products were electrophoresed on a 2% agarose gel. The appropriate bands corresponding to the standard cRNA product and the target mRNA product were excised from the gel and the incorporated radioactivity in each was determined using a scintillation counter. The number of molecules of the target mRNA were determined by extrapolation using the cRNA standard curve. The concentration of PAI-1 mRNA was calculated and expressed as picogram mRNA per microgram total tissue RNA. Variations in sample loading were assessed by measuring β-actin mRNA.

Statistical analysis

All statistical analyses were performed with STATA ver.7 software (STATA Corp., College Station, TX, USA). Comparison of all quantitative RT-PCR results between two age groups (obese vs. lean) was performed with the two-sample *t*-test. Welch's method was applied when variance between two-group was unequal. The *P*-value < 0.05 was considered statistically significant.

In situ *hybridization*

In situ hybridizations for PAI-1 mRNA were performed using riboprobes as described previously [16,26]. After hybridization, the slides were dehydrated by immersion in a graded alcohol series containing 0.3 M NH_4Ac, and dried. The slides were then coated with NTB2 emulsion (Kodak, Rochester, NY, USA; 1:2 in water), and exposed in the dark at 4 °C for 8–12 weeks. The slides were developed for 2 min in D19 developer (Kodak), fixed, washed in water and counterstained with hematoxylin

and eosin. No specific hybridization signal could be detected in parallel sections using ^{35}S-labeled sense probes in each experiment (not shown).

Fibrin immunohistochemistry

Immunohistochemical staining was performed using the Histostain-SP Kit (Zymed Labs., South San Francisco, CA, USA), as described previously [16,26]. Briefly, the tissue sections were deparaffinized, treated with 2% hydrogen peroxide, and incubated with 10% normal goat serum for 30 min. The slides were then incubated with 10 μg mL^{-1} of rabbit anti-mouse fibrinogen/fibrin antibody (a kind gift of Dr E. Plow, Cleveland Clinic), containing 0.1% goat serum at 4 °C overnight, followed by incubation for 1 h at 25 °C. In control experiments, tissues were incubated with preimmune (normal) rabbit IgG instead of the primary antibody. The slides were then washed and treated sequentially with biotinylated goat anti-rabbit IgG (Zymed Labs.), streptavidin-peroxidase conjugate (Zymed Labs.) and aminoethylcarbazole chromogen containing 0.03% hydrogen peroxide (Zymed Labs.). After rinsing in distilled water, the slides were counter-stained with Gill's modified hematoxylin. The specificity of the antibody for fibrin in the extensively perfused tissues was indicated by the absence of staining in tissues from control mice (not shown). Quantitative evaluation of fibrin was achieved by counting the number of glomeruli positive for fibrin in each kidney section (magnification, ×400) in a blinded fashion.

Results

Induction of PAI-1 expression by restraint stress in obese and lean mice

We performed restraint experiments using genetically obese mice and their lean counterparts, and then analyzed PAI-1 expression in their blood and tissues (Fig. 1). Basal (no stress) levels of PAI-1 antigen in plasma and PAI-1 mRNA expression in the adipose tissue were significantly elevated in the obese mice as compared with the lean mice. Interestingly, the induction of PAI-1 antigen in plasma and PAI-1 mRNA expression in the tissues after restraint stress was pronounced in the obese mice. Moreover, in contrast to the lean mice, plasma PAI-1 antigen and adipose PAI-1 expression in the obese mice was dramatically induced by short-duration (2 h) stress. Most importantly, PAI-1 mRNA expression in the hearts was unchanged after restraint stress in the lean mice, but was significantly (threefold) increased in the obese mice. Finally, only short-duration stress induced renal PAI-1 mRNA in the lean mice, while both short- and long-duration stress

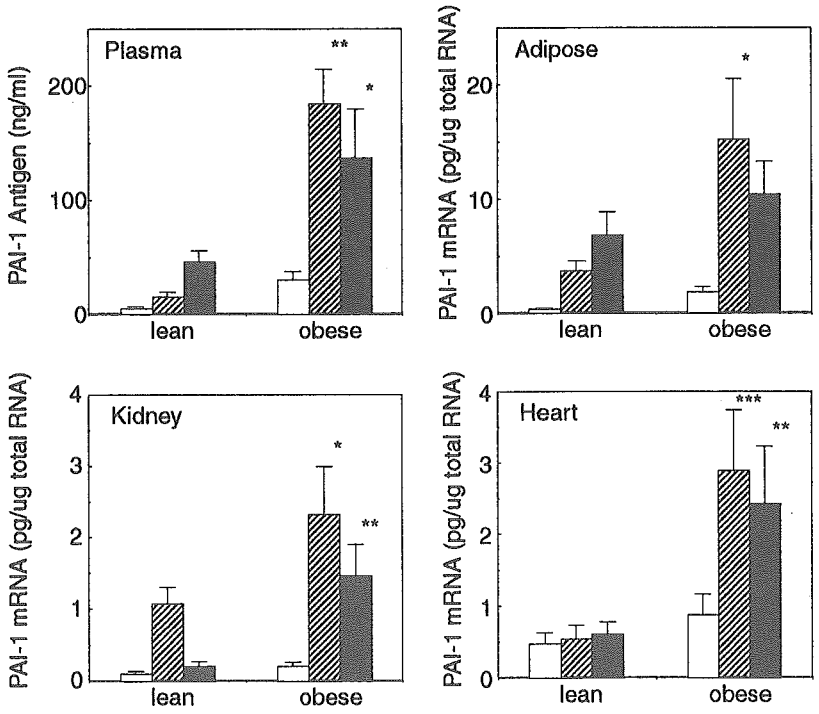

Fig. 1. PAI-1 antigen in plasma and mRNA in tissues after restraint stress in obese and lean mice. Six-week-old C57BL/6J ob/ob mice and their lean counterparts, C57BL/6J +/? mice, were placed into restraint tubes for 2 or 20 h, and then, the blood and tissues were removed. PAI-1 antigen levels in plasma (ng mL^{-1}) were measured by ELISA assay as described in Materials and methods. Total tissue RNA was prepared and analyzed for PAI-1 mRNA expression level by quantitative RT-PCR assay as described in Methods. Open bars, no stress; hatched bars, 2 h-restraint stress; closed bars, 20 h-restraint stress. The data are represented as the mean and SD (n = 8) in each category, and the error bars represent inter-animal variation. *P < 0.05 in obese vs. lean; **P < 0.02 in obese vs. lean; ***P < 0.01 in obese vs. lean.

Table 1 Plasminogen activator inhibitor-1 (PAI-1) antigen levels in tissue extracts of obese and lean mice after restraint stress (all data are presented as picogram of PAI-1 per milligram of tissue, mean ± SE. $n = 8$)

	Before stress		2 h-stress		20 h-stress	
	Obese	Lean	Obese	Lean	Obese	Lean
Adipose	44 ± 8.6	18 ± 3.8	436 ± 83	94 ± 17	409 ± 71	165 ± 31
Kidney	28 ± 2.7	23 ± 2.5	237 ± 33	149 ± 16	168 ± 19	35 ± 2.9
Heart	78 ± 14	69 ± 9.1	252 ± 55	74 ± 15	211 ± 46	77 ± 20

remarkably did so in the obese mice. The magnitudes of the stress-induced PAI-1 mRNA expression in other tissues, including the liver, lung and aorta, were similar in the lean and the obese mice (data not shown).

We examined the PAI-1 expression at antigen level as well in each tissue extract of obese and lean mice after restraint stress (Table 1). In general, large increases in PAI-1 antigen were observed in tissue extracts of the stressed obese mice, which showed similar kinetics with the mRNA level. A larger induction of PAI-1 antigen was shown in the kidneys, hearts, and adipose tissues of obese mice as compared with that in the lean mice. Especially, the adipose-derived PAI-1 antigen induced by stress was about 10-fold higher than basal level, mostly contributing to a marked increase in plasma PAI-1 in the stressed obese mice.

Cellular localization of PAI-1 mRNA in the tissues of stressed obese mice

In control (i.e. before stress) epididymal fat tissues, a few adipocytes were slightly positive for PAI-1 mRNA in both the obese and lean mice (Fig. 2A,E). After a 2-h period of restraint stress, the adipocytes specifically expressing considerable amounts of PAI-1 mRNA increased in both the obese and the lean mice (Fig. 2B,F). The adipocyte-specific signals for PAI-1 mRNA were stronger in the obese mice than those in the lean mice (compare Fig. 2D,H). In the restraint-stressed obese mice, the adipocytes of larger size specifically produced abundant PAI-1 mRNA, and the signals were still considerably strong after a 20-h period of stress (Fig. 2G). In contrast, the signals for PAI-1 mRNA in the epididymal fat tissues of lean mice exposed to 20 h of stress had increased in comparison with those in mice exposed to 2 h of stress (compare Fig. 2B,C).

In the hearts, only focal signals for PAI-1 mRNA were localized in the cardiovascular cells in unstressed obese mice, and few signals were detected in unstressed lean mice (Fig. 3, left panels). However, increased signals for PAI-1 mRNA were observed in some cells (which seemed to be the endothelia of the microvessels lying between the cardiomyocytes) of obese mice exposed to 2 or 20 h of restraint stress, but not in the hearts of stressed lean mice (Fig. 3, middle and right panels).

There was no detectable signal for PAI-1 mRNA in the kidneys of unstressed mice (Fig. 4, left panels). However, some glomerular cells of stressed obese mice expressed considerable amounts of PAI-1 mRNA, while only weak signals for PAI-1 mRNA were detected in the kidneys of stressed lean mice (Fig. 4, right panels). These results are consistent with the data obtained by quantitative RT-PCR assay (see Fig. 1).

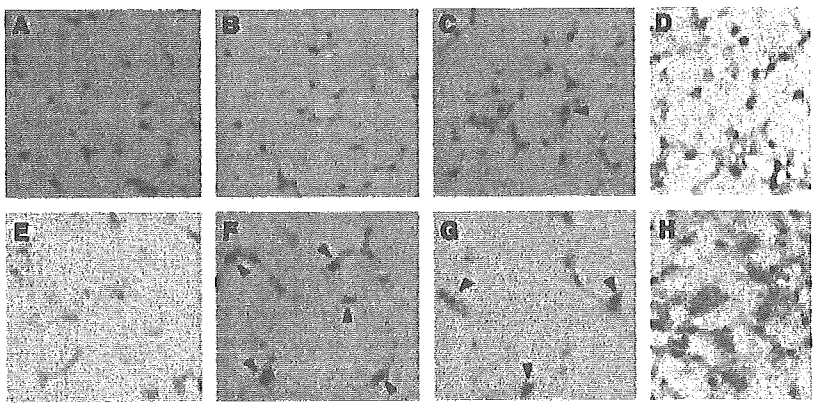

Fig. 2. *In situ* hybridization analysis of PAI-1 mRNA in adipose tissues of the stressed obese and lean mice. Epididymal fat tissues were harvested from 6-week-old obese and lean mice before and after 2 or 20 h-restraint stress and analyzed by *in situ* hybridization using ^{35}S-labeled cRNA probes as described in Materials and methods. The hybridization signal for PAI-1 mRNA corresponds to black dots in panels A–C, E–G (high magnification, ×400) and to light blue dots in panels D, H (low magnification, ×200). Panels A–D: adipose tissues of lean mice (A, no stress; B and D, 2 h-stress; C, 20 h-stress). Panels E–H: Adipose tissues of obese mice (E, no stress; F and H, 2 h-stress; G, 20 h-stress). Arrowheads indicate strongly positive cells for PAI-1 mRNA. All slides were exposed for 8 weeks at 4 °C.