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Diurnal Variation of Fibrinolytic Response to Venous Occlusion

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Summary. **The fibrinolytic system has a circadian variation which has been attributed to changes in plasma concentrations of the tissue plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (PAI-1). PAI-1 has two forms, an active form and latent form; the active form PAI-1 rapidly inactivates t-PA by forming a t-PA/PAI-1 complex. In order to investigate the diurnal variation of the fibrinolytic response of endothelial cells, plasma levels of t-PA antigen, plasminogen activator (PA) activity, PAI-1 antigen, plasminogen activator inhibitor (PAI) activity, and t-PA/PAI-1 complex were measured before and after venous occlusion at 8 A.M. and 4 P.M. in eight healthy subjects. The venous occlusion was performed on the arm at the mean pressure and plasma samples were obtained from the antecubital vein. The basal levels of PAI activity, PAI-1 antigen and t-PA/PAI-1 complex were relatively high in the morning, and the basal PA activity was relatively low in the morning. After venous occlusion, t-PA antigen and t-PA/PAI-1 complex were more increased in the morning than in the evening. However, the levels of PA activity, PAI activity, and PAI-1 antigen similarly increased after venous occlusion both in the morning and in the evening. These findings indicate that, although the basal fibrinolytic activity is low due to the elevation of PAI in the morning, the fibrinolytic response to venous occlusion is similar to that observed in the evening.**

INTRODUCTION

Previous studies have demonstrated that the fibrinolytic activity shows a diurnal variation, with the lowest activity during the early morning hours both in healthy individuals and patients with thrombotic diseases.[1-3] The decreased fibrinolytic activity in the morning has been attributed mainly to the relatively high levels of plasminogen activator inhibitor 1 (PAI-1), which regulates plasminogen activator (PA) activity.[3-7] Muller et al.[8,9] have shown that the time of onset of acute myocardial infarction and sudden cardiac death has circadian variations, and that these variations are similar to the circadian rhythm of plasma PAI-1 concentration. The increased thrombotic tendency resulting from the PAI-1 elevation may be related to the onset of thrombotic diseases.[3,10] The purpose of this study is to investigate whether or not the fibrinolytic response of endothelial cells to venous stimuli shows a diurnal variation. Plasma levels of fibrinolytic parameters such as PA activity, tissue plasminogen activator (t-PA) antigen, plasminogen activator inhibitor (PAI) activity, PAI-1 antigen, and t-PA/PAI-1 complex were measured in healthy subjects before and after venous occlusion in the morning and evening.

MATERIALS AND METHODS

Subjects and blood sampling

The fibrinolytic response was evaluated in eight healthy men (mean age; 28 years, range; 26-32 years) at 8 A.M. and 4 P.M. After blood samples were obtained from the right antecubital vein, venous occlusion (at mean blood pressure) was performed on the left arm for 10 min and then post venous occlusion samples were obtained from the left antecubital vein with the blood pressure cuff still inflated. Blood samples were drawn into siliconized tubes containing either 0.07 volume of 0.129 M trisodium citrate, plus 0.33 volume of 1.0 M sodium acetate buffer (pH 3.9) or 0.1 volume of 0.129 M trisodium citrate. Blood samples were immediately cooled on ice, and centrifuged at 2,000 g for 20 min at 4°C, and plasma was quickly frozen and stored at −70°C until use. In addition, EDTA-anticoagulated blood was prepared for the

determination of hematocrit (Hct).

Assay methods

PA activity was measured in acidified plasma samples by a spectrophotometric parabolic rate assay (Spectrolyse/fibrin; Biopool A.B., Umea, Sweden).[11,12] PAI activity in citrated plasma was measured by a spectrophotometric assay (Spectrolyse/pl(vl-1); Biopool A.B.),[13] and results were expressed in t-PA inhibiting units per ml. The concentrations of t-PA antigen and PAI-1 antigen were determined in citrated plasma samples by sandwich ELISA assay (TintElize t-PA and TintElize PAI-1; Biopool A. B.).[14,15] Plasma t-PA/PAI-1 complex was measured by a two-step sandwich ELISA (TDC-88; Teijin Ltd. Tokyo, Japan). 400 μl of diluted plasma samples and polystyrene balls coated with monoclonal anti-PAI-1 antibody were mixed in test tubes and incubated at 37°C for 1 h. After washing, 400 μl of peroxidase-conjugated polyclonal anti-t-PA antibody was added and incubated at 37°C for 30 min. After washing, the tubes were filled with a substrate solution. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 1 ml of 3 M sulfuric acid and the absorbance was read with a spectrophotometer at 450 nm.

Statistical analysis

Data are reported as mean±SD. Values obtained after venous occlusion were corrected by the following formula: Plasma levels=measured levels×(Hct of pre-venous occlusion/ Hct of post-venous occlusion). Differences between groups were evaluated by the Student's t-test. Regression analysis was performed by the method of least squares, and the correlation coefficient (r) was calculated. Probability levels less than 0.05 were considered statistically significant.

RESULTS

Basal levels of the fibrinolytic parameters

The mean values of plasma PA activity, t-PA antigen, PAI activity, PAI-1 antigen, and t-PA/PAI-1

Table 1. Plasma levels of fibrinolytic parameters before and after venous occlusion

	8 A.M.		4 P.M.		p 8 A.M. vs 4 P.M.
Resting PA activity (IU/ml)	0.37±0.23	p<0.01	0.58±0.21	p<0.05	<0.05
Post-VOT PA activity (IU/ml)	1.86±1.21		2.60±2.24		NS
ΔPA activity (IU/ml)	1.49±1.05		2.01±2.20		NS
Resting t-PA antigen (ng/ml)	5.4±1.8	p<0.001	4.2±1.4	p<0.05	NS
Post-VOT t-PA antigen (ng/ml)	12.3±2.8		8.2±3.1		<0.01
Δt-PA antigen (ng/ml)	6.9±1.8		4.0±2.3		<0.05
Resting PAI activity (IU/ml)	16.7±6.6	NS	6.9±4.4	NS	<0.01
Post-VOT PAI activity (IU/ml)	15.2±8.4		7.5±5.5		<0.02
ΔPAI activity (IU/ml)	−1.5±5.9		0.7±3.3		NS
Resting PAI-1 antigen (ng/ml)	19.5±6.4	p<0.01	11.8±3.8	p<0.05	<0.01
Post-VOT PAI-1 antigen (ng/ml)	26.9±6.9		19.3±6.9		NS
ΔPAI-1 antigen (ng/ml)	7.4±3.8		7.5±7.4		NS
Resting t-PA/PAI-1 complex (ng/ml)	13.3± 5.0	p<0.05	8.6±3.5	NS	<0.01
Post-VOT t-PA/PAI-1 complex (ng/ml)	27.0±14.9		12.9±8.5		<0.05
Δt-PA/PAI-1 complex (ng/ml)	13.7±11.5		4.3±5.4		<0.02

Means±SD are given. VOT=venous occlusion test. NS=not significant ($p \geqq 0.05$).

complex are shown in Table 1. Plasma levels of PAI activity, PAI-1 antigen, and t-PA/PAI-1 complex were higher at 8 A.M. than at 4 P.M. PA activity was higher at 4 P.M. than at 8 A.M. and t-PA antigen levels did not show any difference between 8 A.M. and 4 P.M.

Fibrinolytic response to venous occlusion

The values of PA activity, t-PA antigen, and PAI-1 antigen increased significantly by venous occlusion, but no difference was found in PAI activity between pre- and post-venous occlusion samples. The t-PA/PAI-1 complex increased after venous occlusion at 8 P.M., although the difference in t-PA/PAI-1 complex levels before and after venous occlusion at 4 A.M. did not reach statistically significant levels (Table 1).

The actual degree of increase in the t-PA antigen and t-PA/PAI-1 complex was larger at 8 A.M. than at 4 P.M. However, the increase in other parameters at 8 A.M. was similar to that seen at 4 P.M.

Correlations between fibrinolytic parameters

There was no significant correlation between resting PA activity and resting t-PA antigen ($r=0.20$, $p=0.45$) or resting PAI activity levels ($r=-0.32$, $p=0.23$). Basal t-PA antigen was positively correlated with basal t-PA/PAI-1 complex levels (Fig. 1). No significant correlation was observed between the increase in PA activity following venous occlusion and basal levels of PA activity ($r=0.40$, $p=0.13$) or basal PAI activity ($r=-0.18$, $p=0.49$). A weak correlation was found between the increase in t-PA antigen and the increase in t-PA/PAI-1 complex ($r=0.47$, $p=0.08$), although it did not reach the statistically significant level.

DISCUSSION

Venous occlusion of a limb, which causes a local increase in plasma t-PA concentration, has been used as a diagnostic procedure to assess the fibrinolytic potential.[12,16,17] Previous studies have shown that a poor fibrinolytic response to venous occlusion is commonly found in patients with thrombotic diseases. An impaired fibrinolytic response may result from a decreased release of t-PA from the endothelial cells or an elevation of basal PAI-1.[18,19] We investigated whether the diurnal variation of the fibrinolytic response to venous occlusion is present in humans.

Basal levels of PA activity but not t-PA antigen were lower in the morning than in the evening. In agreement with previous reports,[6,7,20] PAI activity and PAI-1 antigen were higher in the morning than in the evening. In addition, basal levels of t-PA/PAI-1 complex were relatively high in the morning. Although a decline in PA activity in the morning may be related to the higher levels of PAI-1, no direct correlation was found between basal levels of PA

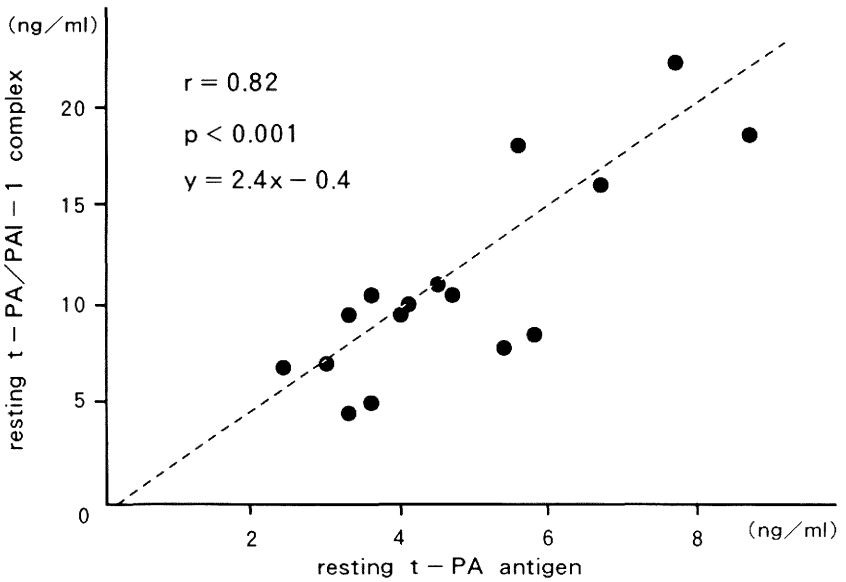

Fig. 1. Correlation between t-PA antigen and t-PA/PAI-1 complex in healthy subjects at rest.

activity and PAI activity in the subjects studied here. In the studies of Angleton et al.,[6] basal PA activity is weakly correlated inversely with PAI activity in healthy subjects and patients with ischemic heart diseases. As the differences between these two findings may be due to differences in the subjects studied, further investigations are required.

After venous occlusion, the levels of PA activity increased and PAI activity did not change (Table 1). The increase in PA activity in the morning was not different from that observed in the evening, indicating that the fibrinolytic response is not poor in the morning as compared with that in the evening. Angleton et al[6] also reported a similar finding in normal subjects and patients with previous myocardial infarction or unstable angina. Furthermore, we demonstrated in this study that the increases in the t-PA antigen and t-PA/PAI-1 complex after venous occlusion were larger in the morning than in the evening, indicating that a larger amount of t-PA is released from the endothelial cells but is inactivated by PAI-1 in the morning.

In conclusion, plasma PAI-1 levels are higher and baseline fibrinolytic activity is suppressed in the morning, but the capacity of fibrinolytic response to venous occlusion is not decreased in the morning compared with that in the evening in healthy subjects. Although frequencies of the onset of thrombotic diseases increase in the morning,[9,10] there is no evidence that the elevation of PAI-1 directly triggers these diseases. Additional studies in patients with higher levels of basal PAI-1 are needed to elucidate further the relationship between the diurnal variation of the fibrinolytic system and the onset of thrombotic diseases.

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