Circadian Variation in Plasma Levels of Free-Form Tissue Factor Pathway Inhibitor Antigen in Patients With Coronary Spastic Angina

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Tissue factor pathway inhibitor (TFPI) is known to inhibit the initial reaction in the tissue factor-mediated coagulation pathway. We measured plasma free-form TFPI antigen levels and monitored 24-h Holter recordings at 06.00, 14.00 and 22.00 h in 15 patients with coronary spastic angina, 13 patients with stable exertional angina, and 11 control subjects. There was a significant circadian variation in plasma free-form TFPI antigen levels in patients with coronary spastic angina (25.8 ± 2.0 ng/ml at 06.00 h, 21.1 ± 1.6 ng/ml at 14.00 h, and 20.2 ± 1.4 ng/ml at 22.00 h; $p < 0.01$). Furthermore, free-form TFPI antigen levels at 06.00 h were significantly higher in coronary spastic angina patients than in patients with stable exertional angina or control subjects ($p < 0.01$). Free-form TFPI antigen levels increased after the ischemic attacks in coronary spastic angina ($p < 0.01$). This circadian variation correlated with the frequency of attacks, with the peak level occurring between midnight to early morning in patients with coronary spastic angina. (*Jpn Circ J* 1998; 62: 419–424)

Key Words: Free-form tissue factor pathway inhibitor; Circadian variation; Coronary spastic angina; Ischemic attack

Coronary spastic angina is caused by coronary artery spasm^{1,2} which has also been implicated in the pathogenesis of unstable angina^{3,4} and acute myocardial infarction^{5,6}. Coronary thrombosis plays a pivotal role in the genesis of unstable angina and acute myocardial infarction^{7–9}. An increased risk of thrombosis arises for several reasons, such as increased platelet aggregation¹⁰, activation of the coagulation system^{11–13} or a defective fibrinolytic system¹⁴. We have previously reported significant circadian variations in the levels of plasma fibrinopeptide A (FPA), a specific marker for thrombin generation¹¹ and the activity of plasminogen activator inhibitor (PAI), which is an indicator of defective fibrinolysis, in patients with coronary spastic angina^{15–18}.

Tissue factor pathway inhibitor (TFPI) is a protease inhibitor with 3 tandem Kunitz-type (K1, K2, and K3) domains¹⁹ that inhibits the initial reaction of the tissue factor-mediated coagulation pathway by forming complexes with factor Xa, factor VIIa, and tissue factor^{20,21}. TFPI exists both as a free form and as a lipoprotein-associated form that binds to low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein(a) [Lp(a)] in the plasma^{22,23} and which acts as an endothelial cell-associated form on vascular walls²¹. It has

recently been shown that TFPI is stored intracellularly within endothelial cells in culture and is redistributed to the cell surface on acute stimulation with thrombin²⁴. Plasma levels of TFPI increase substantially and rapidly after administration of heparin. The increase in TFPI in post-heparin plasma may be caused by the release of TFPI from the surface of the vascular endothelium²⁵.

In this study, we examined whether there is a circadian variation in the level of the free form of TFPI in patients with coronary spastic angina. We also evaluated the relationship between the circadian variation in the plasma free-form TFPI antigen and ischemic attacks.

Methods

Patient Population

Thirty-nine patients [30 men and 9 women, age 62.9 ± 1.6 years (mean $\pm$ SEM), range 39–78 years] who underwent diagnostic cardiac catheterization were studied. They were divided into 3 groups: the coronary spastic angina, stable exertional angina, and control groups.

The coronary spastic angina group consisted of 15 patients (13 men and 2 women, age 60.5 ± 2.5 years, range 43–78 years). All patients had attacks of chest pain accompanied by ST-segment elevation or depression of the standard 12-lead electrocardiogram (ECG) at rest, usually occurring between midnight and early morning. No patient exhibited ST-segment changes lasting more than 30 min, new Q waves, or raised plasma concentrations of creatine kinase or creatine kinase MB.

The stable exertional angina group consisted of 13 pa-

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Table 1 Characteristics of the Study Groups

Characteristics	Coronary spastic angina	Stable exertional angina	Controls
No. of patient	15	13	11
Age			
Mean	60.5 (2.5)	64.9 (2.9)	63.6 (2.7)
Range	43–78	39–77	50–77
Men/Women	13/2	11/2	6/5
Previous myocardial infarction (n)	0	9	0
Hypertension (n)	5	5	5
Smokers (n)	11	7	4
Diabetes melitus (n)	1	1	1
Obesity (n)	3	5	2
Serum total cholesterol (mg/dl)	203 (9)	202 (7)	206 (10)
Serum LDL-cholesterol (mg/dl)	132 (7)	129 (7)	122 (7)
Serum HDL-cholesterol (mg/dl)	45 (4)	46 (4)	56 (6)
Serum triglyceride (mg/dl)	121 (11)	130 (18)	131 (26)
Extent of coronary vessel organic stenosis $\geq 75\%$ stenosis			
0-vessel	8*	0	11
1-vessel	5	8	0
2-vessel	2	3	0
3-vessel	0	2	0
Coronary artery spasm (n)			
Single vessel spasm	6	0	0
Multivessel spasm	9	0	0
Ejection fraction $< 50\%$ (n)	1	3	0
Medication used (n)			
β -blocker	0	3	0
Long acting nitrates	2	6	0
Calcium antagonist	4	10	3
Aspirin	0	5	1

Values are means (SEM). * $p < 0.01$ vs stable exertional angina. All drugs except nitroglycerin were stopped for at least 3 days before the study day. However, one patient with coronary spastic angina who had severe attacks was treated with calcium antagonist.

tients (11 men and 2 women, age 64.9 ± 2.9 years, range 39–77 years) who had typical chest pain on exertion associated with horizontal or down-sloping ST-segment depression of greater than 1.0 mm at 80 msec after the J point on an exercise test. All patients in this group had no episodes of rest angina and had on angiography exhibited advanced organic stenosis in at least coronary artery 1 and involving at least 90% of the luminal diameter. The most recent attack in each patient of this group had occurred 4 or more weeks before entry into the study. No change in the frequency of angina, precipitating factors, or responses to sublingual nitroglycerine had been noted in the past 3 months in this study group.

The control group consisted of 11 patients (6 men and 5 women, age 63.6 ± 2.7 years, range 50–77 years) with atypical chest pain unaccompanied by electrocardiographic changes of ischemia on a treadmill exercise test and a hyperventilation test. No patient had significant stenosis ($< 25\%$ stenosis of the luminal diameter) on coronary arteriography.

The stable exertional angina group and the control group were selected from patients undergoing elective cardiac catheterization during the same period and who had been matched for age, gender, and other clinical variables with the coronary spastic angina group. Table 1 shows the clinical characteristics of the 3 groups.

None of the patients was treated with thrombolytic agents such as urokinase, streptokinase, or alteplase, or

anticoagulants or steroids. None of them had thromboembolism, collagen disease, disseminated intravascular coagulation, advanced liver disease, renal failure, malignant disease, septicemia, or other inflammatory diseases. None of them had prosthetic heart valves or pacemakers.

Cardiac catheterization including coronary arteriography was performed in all patients within a week after the study. Coronary arteriography showed coronary artery spasm during the attacks of angina provoked by intracoronary injection of acetylcholine (50 μ g or 100 μ g) in all 15 patients with coronary spastic angina. Five patients with coronary spastic angina had significant organic stenosis involving 75% or more of the luminal diameter in one major coronary artery. Two patients had 2-vessel disease. None of the control subjects had significant organic stenosis in their coronary arteries, and coronary artery spasm was not induced by intracoronary injection of acetylcholine (50 μ g or 100 μ g) in any of the control subjects. Table 1 shows the number of diseased vessels in the 3 groups.

Study Protocol

Blood samples were obtained from patients at 06.00, 14.00, and 22.00 h within 1 week the day before cardiac catheterization. All patients underwent Holter ECG (Der Mar Avionics, Irvine, CA, USA) with a model 447 2-channel recorder for at least 24 h on the day of blood sampling. An ischemic attack was defined as more

than 2.0 mm ST-segment elevation or more than 1.0 mm horizontal or down-sloping ST-segment depression 80 msec after the J point that lasted for at least a minute. We took blood samples before (at 22.00 or 06.00 h) and immediately after ischemic attacks from 05.00 to 08.00 h in addition to routine samplings in 9 patients with coronary spastic angina. We took blood samples before and immediately after ischemic attacks induced by treadmill exercise from 06.00 to 08.00 h in 9 patients with stable exertional angina on the another day.

The study protocol was in agreement with the guidelines of the ethics committee at our institution. Informed consent was obtained from each patient.

Blood Sampling

Fasting blood samples were obtained from each patient using a 21-gauge needle for clean venipuncture of an antecubital vein without stasis by specially trained physicians (KM and HS). The sites of venipuncture of each patient were changed each time because there was the possibility that slight residual hematomas would confound some of the data. The 2.7 ml of blood required for the free-form TFPI antigen assay was drawn into a tube containing sodium citrate (0.13 mol/L, pH 7.5). The samples for the free-form TFPI antigen assay were immediately centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, the samples of plasma were stored immediately at -80°C until used.

Determination of Free-Form TFPI Antigen Levels

Plasma free-form TFPI antigen levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit. Results were expressed as ng/ml. The assay, which uses monoclonal antibodies raised against free-form TFPI, has been shown to be specific, sensitive, reproducible, and appropriate for routine clinical application. The kit for the plasma free-form TFPI antigen was produced by the Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN; Kumamoto, Japan). It used 2 different monoclonal antibodies against TFPI obtained by the cell fusion method^{26,27}. One monoclonal antibody immobilized to the solid phase (microplate well) recognized the third Kunitz domain²⁶ and the other monoclonal antibody, which conjugates with horse radish peroxidase (HRP), recognized the specific conformation formed between the first and second Kunitz domains²⁷. Assay procedures for this kit were as follows: after the addition of 100 µl of the HRP-labeled anti-TFPI monoclonal antibody and 100 µl of plasma samples to the microplate wells coated with the anti-TFPI monoclonal antibodies, the microplate was incubated for 2 h at 37°C. The plate was washed 6 times with the washing buffer contained in the kit, and 200 µl of the substrate solution 3, 3', 5, 5'-tetramethylbenzidine and H₂O₂ was dispensed into each well, and the plate was then incubated for 30 min at 37°C. The enzyme reaction was stopped by adding the stop solution (0.5 mol/L sulfuric acid) provided in the kit. Absorbance at 450 nm was measured with a microplate reader M-Emax (Wako, Osaka, Japan). The amounts of TFPI antigen were calculated from a standard curve obtained with human recombinant TFPI standards provided in this kit. Inter-assay and intra-assay coefficients of variation of TFPI antigen levels of 12.5 ng/ml using this kit were 10.3% and 9.6%, respectively.

Statistical Analysis

All data are shown as means ± SEM. The changes in plasma free-form TFPI antigen levels among the 3 groups were compared employing repeated-measures analysis of variance (ANOVA). When these results were statistically significant, we used the Bonferroni criterion. Age, serum cholesterol (total cholesterol, HDL-cholesterol, LDL-cholesterol), and serum triglyceride levels were compared by one-way ANOVA. When these results were statistically significant, they were analyzed by Scheffé's F test for multiple comparisons. The clinical characteristics of the 3 groups shown in Table 1, except age, serum cholesterol, and serum triglyceride, were compared by a chi-square test. The distribution of the frequency of ischemic attacks during 3 periods of 8 h was tested for differences using the Friedman test. Changes in plasma free-form TFPI antigen levels before and after ischemic attacks were compared by a paired t test. A probability value of <0.05 was considered statistically significant.

Results

Characteristics of the Study Groups

Table 1 shows the clinical characteristics of patients with coronary spastic angina, patients with stable exertional angina, and control groups on admission. The medications taken by patients in the coronary spastic angina and stable exertional angina groups (β -blockers, long-acting nitrates, calcium antagonists, aspirin) are also shown in Table 1. The level of free-form TFPI may be influenced by age, sex, hyperlipidemia [LDL-cholesterol, HDL-cholesterol, triglyceride, Lp(a)], diabetes mellitus, hypertension, smoking, and obesity. There were no significant differences in these variables among the 3 study groups. Four patients with stable exertional angina had a history of old myocardial infarction at least 3 months before the study. In principle, all drugs except nitroglycerin were stopped for at least 3 days before the study day. However, 1 patient with coronary spastic angina had severe attacks and was treated with anti-anginal drugs (calcium antagonist) on the day of the study. Four of the 13 patients with stable exertional angina were receiving medication on the day of the study: 3 were receiving long-acting nitrates and aspirin and 1 patient was receiving a calcium antagonist, long-acting nitrates, a β -blocker, and aspirin because of a history of unstable angina and multiple-vessel disease with severe organic stenosis. None of the control subjects was receiving medication on the day of the study.

Results of 24-h Holter ECG

Thirteen patients with coronary spastic angina had between 1 and 16 ischemic attacks, and 118 of these episodes occurred on the day of the study. Two patients with coronary spastic angina had no ischemic attacks on the day of the study. Twenty-three (19%) of the attacks occurred between 14.00 and 22.00 h, 62 (53%) occurred between 22.00 and 06.00 h, and 33 (28%) occurred between 06.00 and 14.00 h. There was a significant circadian variation in the frequency of attacks, which reached a peak between 22.00 and 06.00 h ($p < 0.01$) (Fig 1). The patients with stable exertional angina and control subjects had no attacks during the study.

Plasma Free-Form TFPI Antigen Level

Fig 1. Circadian distribution of the number of ischemic attacks in patients with coronary spastic angina. There were significant circadian variations in the frequency of attacks, which reached a peak between 22.00 and 06.00 h ($p < 0.01$).

Fig 2. Plasma free-form TFPI antigen levels in patients with coronary spastic angina, patients with stable exertional angina, and control subjects. Data are expressed as means (SEM). Plasma free-form TFPI antigen levels showed a significant circadian variation, with a peak at 06.00 h, only in patients with coronary spastic angina ($p < 0.01$). Plasma free-form TFPI antigen levels at 06.00 h were significantly higher in patients with coronary spastic angina than in stable exertional angina patients or control subjects ($p < 0.01$).

Fig 2 shows the mean free-form TFPI antigen levels in the 3 study groups at 06.00, 14.00, and 22.00 h. There were significant circadian variations in plasma free-form TFPI antigen levels only in the coronary spastic angina

Fig 3. Line graph showing plasma free-form TFPI antigen levels before (at 22.00 or 06.00 h) and immediately after 9 ischemic attacks in 9 patients with coronary spastic angina. Plasma free-form TFPI antigen levels were increased significantly immediately after the ischemic attacks as compared with before the attacks ($p < 0.01$).

Fig 4. Line graph showing plasma free-form TFPI antigen levels before and after 9 ischemic attacks in 9 patients with stable exertional angina by treadmill test on the another day. Plasma free-form TFPI antigen levels were not increased significantly immediately after the ischemic attacks as compared with before the attacks.

group. The level of TFPI was significantly higher at 06.00 h (25.8 ± 2.0 ng/ml, $p < 0.01$) than at 14.00 h (21.2 ± 1.6 ng/ml) and at 22.00 h (20.2 ± 1.4 ng/ml) in patients with coronary spastic angina (Fig 2). Furthermore, the level

was significantly higher (25.8 ± 2.0 ng/ml, $p < 0.01$) in patients with coronary spastic angina than in patients with stable exertional angina (18.2 ± 1.8 ng/ml) and in the control groups (16.9 ± 1.4 ng/ml) at 06.00 h (Fig 2). These circadian variations correlated with the frequency of attacks, with the peak level occurring from midnight to early morning in patients with coronary spastic angina.

Plasma Free-Form TFPI Antigen Levels Before and Immediately After Ischemic Attacks

We took blood samples to examine plasma free-form TFPI antigen levels before (at 22.00 or 06.00 h) and immediately after 9 ischemic attacks in 9 patients with coronary spastic angina. Plasma free-form TFPI antigen levels increased significantly immediately after the ischemic attacks as compared with before the attacks (Fig 3).

Plasma Free-Form TFPI Antigen Levels Before and Immediately After Ischemic Attacks Induced by Treadmill Exercise

We took blood samples to examine plasma free-form TFPI antigen levels before and immediately after 9 ischemic attacks induced by treadmill exercise in 9 patients with stable exertional angina on the another day. Plasma free-form TFPI antigen levels were not increased significantly immediately after the ischemic attacks as compared with before the attacks (Fig 4).

Discussion

Coronary artery spasm is significantly implicated in the genesis of unstable angina and acute myocardial infarction in some patients.³⁻⁶ We have reported that plasma fibrinopeptide A (FPA) levels and plasminogen activator inhibitor (PAI) activity were increased after coronary spasm.¹⁵⁻¹⁷ We have also shown that there are significant circadian variations in FPA levels and PAI activity correlated with the frequency of the attacks, with the peak level occurring from midnight to early morning in patients with coronary spastic angina.^{15,17}

TFPI inhibits the initial reaction in the tissue factor-mediated coagulation pathway.¹⁹⁻²¹ In addition, Kokawa et al²¹ have shown that the level of free-form TFPI antigen in plasma is positively correlated with the level of endothelial cell-released TFPI antigen, and that the level of free-form TFPI antigen in plasma generally reflects the level of endothelial cell-associated TFPI. In this study, there were significant circadian variations in plasma free-form TFPI antigen levels only in the coronary spastic angina group, with the peak level occurring at 06.00 h. Furthermore, the level of plasma free-form TFPI antigen at 06.00 h was significantly higher in the coronary spastic angina group than in the stable exertional angina and the control groups. The level of plasma free-form TFPI antigen increased immediately after ischemic attacks in patients with coronary spastic angina. There were significant circadian variations in TFPI antigen levels correlated with the frequency of the attacks, with the peak level occurring between 22.00 and 06.00 h in patients with coronary spastic angina. There were no significant differences in the free-form TFPI antigen levels in the stable exertional angina and control groups at any sampling time.

Recently, to determine whether coronary spasm affects plasma TFPI levels, we measured the heparin-releasable

endothelial cell-associated TFPI in the coronary sinus and the aortic root before and after coronary spasm induced by an injection of acetylcholine in patients with coronary spastic angina. The results showed that heparin-releasable endothelial cell-associated TFPI levels in the coronary spastic angina group significantly increased in the coronary sinus after the ischemic attack but not in the aortic root.²⁸ These results suggested that heparin-releasable endothelial cell-associated TFPI is increased in coronary circulation after coronary spasm.

We have reported that thrombin generation after coronary spasm may result in the release of FPA and PAI from the coronary circulation.^{16,17} Thrombin potently stimulates endothelial cells and affects the production or secretion of various mediators such as von Willebrand factor, tissue plasminogen activator, and PAI and TFPI, which are involved in thrombosis and fibrinolysis.^{24,29-31} Thrombin generation after coronary spasm may induce the release of TFPI from endothelial cells into the plasma. It is thought that the circadian variation in plasma free-form TFPI antigen levels correlates with the circadian variation in plasma levels of FPA, a specific marker for thrombin generation. In addition, we believe that coronary spasm can induce an acute inflammatory reaction in the coronary circulation because soluble P-selectin is released into the coronary circulation during coronary spasm accompanied by myocardial ischemia.³² It is possible that acute inflammatory reactions after coronary spasm may lead to increased TFPI release into the plasma.

TFPI has been reported to be increased in patients with acute myocardial infarction and angina pectoris³³ and decreased in patients with ischemic stroke or hypercholesterolemia, reflecting the reduced antithrombotic properties of vascular endothelial cells.^{21,26,34} It is important to examine free-form TFPI levels to diagnose these pathophysiologic conditions in patients.²¹ Recently, it has been reported that inhibition of the coagulation system by administration of recombinant TFPI prevents reocclusion and sustains patency of arteries recanalized by pharmacologic fibrinolysis in animal models.^{35,36} These results indicate that inhibition of activation of the coagulation pathway in patients with coronary spastic angina is subject to significant circadian variation.

This study has shown that there are significant circadian variations in plasma levels of free-form TFPI antigen, with the peak level occurring in the early morning in patients with coronary spastic angina. It is thought that endothelial-cell associated TFPI is increased by anginal attacks, with the peak level occurring in the early morning in patients with coronary spastic angina. In conclusion, free-form TFPI may be released from endothelial cells into the plasma after coronary spasm.

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