DETECTION OF IMPAIRED FIBRINOLYTIC ACTIVITY IN CORONARY ARTERY DISEASE —Electrophoretic and Immuno-Blotting Analysis of Tissue Plasminogen Activator—

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In present study, we investigated the fibrinolytic activities and plasma antigen levels of tissue plasminogen activator (tPA) before or after a submaximal exercise in patients with coronary artery disease (CAD). We also investigated tPA phenotypes in plasma by electrophoretic and immuno-blotting analysis. Euglobulin fractions obtained from plasma were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immuno-blotting analysis.

There were no differences in plasma antigen levels of tPA between the study group and controls before or after the exercise, however CAD patients showed lower fibrinolytic activities after the exercise than controls. SDS-PAGE followed by immuno-blotting with an antisera against human tPA revealed two bands at molecular weights (m.w.) of 70,000 and 120,000. The band at m.w. of 70,000 corresponded to free tPA and that of 120,000 was considered to be identical to a complex of tPA with its inhibitor. Furthermore, we found a decrease in free tPA in the patients with low fibrinolytic activities. From these results it was concluded that impaired fibrinolytic activities, probably due to decreased free tPA, observed in CAD patients, might be an important factor in the pathogenesis of CAD.

Redced fibrinolytic activity is widely known to play an important role in the initiation and development of coronary artery disease (CAD)1–3 Recently, much attention has been given to tissue plasminogen activator (tPA) as one of the important regulatory factors of intravascular thrombosis, because tPA is derived mainly from vascular endothelial cells4–6. It has a higher affinity for fibrin but not for fibrinogen when compared with urokinase7, which is another important plasminogen activator in the blood. However, except for the venous occlusion test (VOT)8 and the DDAVP (1-desamino-8-arginine vasopressin) infusion test9, there are few established tests to evaluate the vascular function of antithrombogenesis. On the other hand, physical exercise has been reported to have a strong enhancing effect on fibrinolytic activity1,10 and the physical exercise stress test is commonly used to evaluate cardiac functions in patients with CAD. Therefore, we employed a

Key words: Coronary artery disease Tissue plasminogen activator Fibrinolytic activity SDS-PAGE with immuno-blotting

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TABLE 1 PATIENTS WITH CORONARY ARTERY DISEASE

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex (M:F)</th>
<th>No. of diseased coronary arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMI</td>
<td>14</td>
<td>54 ± 11</td>
<td>1VD 2VD 3VD</td>
</tr>
<tr>
<td>AP</td>
<td>12</td>
<td>55 ± 11</td>
<td>9 : 3 5 4 3</td>
</tr>
</tbody>
</table>

OMI = old myocardial infarction; AP = angina pectoris; 1VD = 1 vessel disease; 2VD = 2 vessel disease; 3VD = 3 vessel disease

submaximal exercise test on a bicycle ergometer as the stimulus of fibrinolytic activity in this present work.

We investigated fibrinolytic activities and plasma antigen levels of tPA before or after the exercise and also studied tPA phenotypes in plasma in relation to the biological activities of tPA using electrophoretic and immuno-blotting analysis.

MATERIALS AND METHODS

(1) Subjects

i) The patient group consisted of 26 patients with CAD, 14 with old myocardial infarction (OMI), 12 with angina pectoris (AP). A total of 21 were men and 5 were women, aged 39–72 years (55 ± 11; mean ± SD). The number of diseased coronary arteries in the patients with OMI or those with AP is shown in Table I. The diagnosis of CAD was made on the basis of clinical symptoms, electrocardiograms (ECG), two dimensional echocardiograms and coronary cineangiograms (CAG).

ii) The control group (CTR) consisted of 13 apparently healthy subjects, 8 men and 5 women, aged 15–61 years (49 ± 12; mean ± SD).

Both groups were similar in age and had not undergone recent operations or had not been on anticoagulant therapies. The patients with CAD were free from anjinal attacks for at least 2 weeks and had refrained from smoking for 12 hours prior to the exercise test.

As to antianginal medication, isosorbide nitrate was administered to 5 patients and Ca++ antagonists (diltiazem or nifedipine) to 3 patients, and the combination of both to 8 patients. A combination of isosorbide nitrate, Ca++ antagonists and nicorandil was administered to 3 patients, and that of isosorbide nitrate and nicorandil to 2 patients, and that of nicorandil and Ca++ antagonists to 2 patients. The other 2 patients were given β-blockers (propranolol or acebutolol), and 1 patient received no medication.

(2) Exercise testing

Both groups performed a graded submaximal exercise test (25W/3min) on a bicycle ergometer in a symptom-limited manner. The end points of the exercise test were determined by the following criteria: 1) attainment of the individually predicted target heart rate; 2) appearance of typical anginal chest pain; 3) diagnostic ST segment changes in ECG (ST segment elevation or depression more than 0.2 mV); 4) restriction of physical exercise because of dyspnea etc., and 5) elevation of systolic blood pressure over 220 mmHg. As parameters of the exercise capacity of the individuals, exercise time (min), work load (W), heart rate (HR) and blood pressure (BP) at peak exercise and rate pressure products (RPP; HR × systolic BP at peak exercise) were evaluated.

(3) Blood sampling

Blood was collected from antecubital vein before and immediately after the exercise by a two syringe technique with a least venous occlusion, and immediately anticoagulated with 0.1 vol of 3.8% trisodium citrate in a plastic tube. Platelet poor plasma (PPP) was prepared by centrifugation of the sample at 3,000 r.p.m. for 10 min at 0°C and kept frozen at −40°C until use.

(4) Plasma antigen levels of tPA (ng/ml)

Plasma antigen levels of tPA were assayed by an enzyme-linked immunosorbent assay (ELISA).10

(5) Fibrinolytic activities (u/ml)

Euglobulin fractions were prepared from plasma according to C.V. Prowse11 et al. Briefly, 1 vol of PPP was incubated with 19 vol of 0.016% acetic acid in ice bath for 1 hour and centrifugated 3,000 r.p.m. at 0°C for 10 min. The supernatant was aspirated and the residual precipitate was redissolved in a volume of Owren’s veronal buffer with pH 7.35 corresponding to the same initial PPP volume (euglobulin solution). Ten µl of euglobulin solution was applied in duplicate on the fibrin-agar plate. After 20 hours of incubation at 37°C, fibrinolytic zones were calculated and converted into units by compar-
son with a standard preparation of two chain tPA which was a kind gift from Wellcome Corporation (England).

(6) Electrophoretic and immuno-blotting analysis

<table>
<thead>
<tr>
<th></th>
<th>CTR</th>
<th>CAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exercise time (min.)</td>
<td>11.7 ± 3.3</td>
<td>9.9 ± 3.2</td>
</tr>
<tr>
<td>Work load (W)</td>
<td>119.2 ± 37.0</td>
<td>96.8 ± 31.1</td>
</tr>
<tr>
<td>Rate Pressure Products</td>
<td>29098 ± 4887</td>
<td>25731 ± 5238</td>
</tr>
</tbody>
</table>

CTR : control subjects  mean ± SD  CAD : coronary artery disease

There was no significant differences in each parameter between the subjects with coronary artery disease (CAD) and controls (CTR).

Euglobulin fractions before and after the exercise were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli\textsuperscript{12} using a 7.5% separating gel and a 4.5% stacking gel. Twenty µl of the euglobulin solution was incubated in an equal volume of sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 0.02% bromophenol blue) for 1 hour and then located into the gel and subjected to electrophoresis for 7 hours or until the dye reached the bottom of the gel. After electrophoresis, the polyacrylamide gel was equilibrated in transfer buffer (0.025 M Tris-HCl containing 0.192 M glycine and 20% methanol) for 30 min and then placed on Durapore\textsuperscript{R} membrane (Millipore), which was pretreated with 20% methanol for 30 sec, and then equilibrated in the transfer buffer for 30 min. Proteins separated on the gel were transferred to the membrane electrophoretically according to Towbin et al\textsuperscript{13} and then identified by an enzyme immunoassay using double antibodies; the first was a rabbit antibody against human tPA and the second was a peroxidase-

![Graph showing plasma antigen levels of tPA in patients with coronary artery disease (CAD) and controls (CTR)].

Fig.1. shows plasma antigen levels of tPA in patients with coronary artery disease (CAD) and controls (CTR). There was a significant increase in plasma antigen levels of tPA during the exercise in patients with CAD and CTR. But no difference of significance between the groups was observed at rest or after the exercise respectively.

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Fig. 2. shows fibrinolytic activities in patients with coronary artery disease (CAD) and controls (CTR) at rest or after the exercise.

Significant increase was observed during the exercise in both groups, however patients with CAD showed lower fibrinolytic activities after the exercise compared with controls.

Conjugated goat antibody against rabbit Ig G (Cappel). The details of the enzyme immunoassay were as follows: the membrane was blocked by 10% bovine serum albumin (BSA, Sigma) in 0.01 M Tris-HCl pH 7.4 containing 0.9% NaCl (TBS) at 37°C for 3 hours and washed 3 times at 15 min intervals with TBS containing 0.05% Tween 20 (TBS-Tween 20) at room temperature. The membrane was reacted with the first antibody against human tPA at room temperature for 5 hours and then washed 3 times in the same manner. The membrane was reacted with the second antibody against rabbit Ig G at room temperature for 2 hours and then washed 3 times again. The membrane was put into chromophoric solution prepared by a mixture of 30 mg of 4-Cl-1-naphtol in 10 ml of cooled methanol with 50 ml of TBS containing 30 μl of 30% H2O2. The reaction was stopped in distilled water and the bands exhibited on the membrane were photographed.

RESULTS

(1) Exercise testing on a bicycle ergometer

The mean exercise time, work load and rate pressure products (RPP) in the study group were 9.9 ± 3.2 min, 96.8 ± 31.1 W and 25731 ± 5238, respectively and those of controls were 11.7 ± 3.3 min, 119.2 ± 37.0 W and 29098 ± 4887. There were no significant differences between the two groups in each parameter (Table II). The HR and systolic BP at peak exercise were 139.3 ± 19.9/min and 182.9 ± 28.4 mmHg in CAD group, and 155.5 ± 12.4/min and 187.6 ± 31.0 mmHg in the control group respectively. There was a significant difference between the two groups in the HR at peak exercise but not in the peak systolic BP.

(2) Changes in plasma antigen levels of tPA

The mean antigen levels of tPA (mean ± SD) in plasma before and after the exercise were 3.09 ± 1.41 and 3.71 ± 1.49 ng/ml in the study
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Fig. 3. Relationship between fibrinolytic activities and plasma antigen levels of tPA in controls (Fig. 3A) and the patients with CAD (Fig. 3B). An inverse correlation was observed in controls, but there was no significant correlation in the patients.

Fig. 4A. The proteins of euglobulin fraction separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Fig. 4B. Immuno-blotting analysis of euglobulin fractions using an antibody against human tissue plasminogen activator. Left sided 2 lanes were the bands in the subjects with high fibrinolytic activities and right sided 2 lanes were those in the subjects with low fibrinolytic activities.

plasma antigen levels in the patient group rather than in controls. Furthermore, the mean antigen levels of tPA (mean ± SD) in plasma before and after the exercise were 2.80 ± 1.37 and 3.51 ± 1.37 ng/ml respectively in patients with OMI or 3.36 ± 1.45 and 3.90 ± 1.64 ng/ml respectively in those with AP but there was no significant dif-

ference between the patients with OMI and AP.

(3) Changes in fibrinolytic activities

The mean fibrinolytic activities (mean ± SE) at rest were 0.17 ± 0.07 (U/ml) in the study group and 0.53 ± 0.22 (U/ml) in controls (Fig. 2). Significant increase was observed in both groups after the exercise as compared with those before (CAD: 0.80 ± 0.25; controls: 15.67 ± 5.61 U/ml). But patients with CAD showed lower fibrinolytic activities after exercise compared with controls. And the mean fibrinolytic activities (mean ± SE) before and after the exercise were 0.08 ± 0.03 and 0.97 ± 0.40 U/ml in the patients with OMI and 0.29 ± 0.14 and 0.57 ± 0.26 U/ml in those with AP respectively, but there was no significant difference between the two groups of patients.

(4) Relationship between fibrinolytic activities and antigen levels of tPA in plasma

We investigated the correlation between fibrinolytic activities and corresponding tPA antigen levels in plasma before or after the exercise, because the conventionally determined antigen levels of tPA consisted of not only free tPA but also complexed tPA with its inhibitor. There was no positive correlation between fibrinolytic activities and the plasma antigen levels of tPA in both groups, however there was an inverse correlation between them in controls but not in the CAD group (Fig. 3).

(5) Electrophoretic and immuno-blotting analysis

SDS-PAGE of euglobulin fraction revealed 12 visual bands (Fig. 4A). The following immuno-blotting analysis revealed two bands at m.w. of 70,000 and 120,000 (Fig. 4B). The intensity of 120,000 band was much stronger than the 70,000 one. Furthermore, a decrease in the density of the band at m.w. of 70,000 was observed in euglobulin fractions of the patients with low fibrinolytic activities (Fig. 4B, right sided 2 lanes), as compared with those with high fibrinolytic activities (Fig. 4B left sided 2 lanes).

**DISCUSSION**

Reduced fibrinolytic activity has been reported to be responsible for the pathogenesis of deep vein thrombosis and myocardial infarction.\(^{14-15}\) tPA is a physiological and the most important plasminogen activator in the blood with respect to the regulatory mechanism of intravascular thrombosis. In previous reports, euglobulin fraction had been mainly used to measure fibrinolytic activity in plasma, because the direct measurement of its activity was difficult due to the interferences of the coexisted protease inhibitors in plasma. These inhibitors were considered to be solubilized and removed in the process of preparing euglobulin fraction except for C\(_4\) inhibitor.\(^{16}\) However, in the present study electrophoretic and immuno-blotting analysis of euglobulin fraction revealed two bands at m.w. of 70,000 and 120,000. In previous reports on the m.w. of tPA, the 70,000 band corresponded to free tPA,\(^{17-18}\) and the 120,000 one was probably a complex of tPA with its major inhibitor in the blood, plasminogen activator inhibitor type 1 (PAI-1).\(^{17-20}\) It was suggested from these results that most of tPA in euglobulin fractions existed in a complexed form with its inhibitor, PAI-1 and the inhibitor complexed with tPA was recovered in euglobulin fraction.

Recently PAI-1 was proved to be derived mainly from vascular endothelial cells and also from platelets\(^{21}\) and to inactivate tPA immediately when it is released from vascular beds into blood. Now this PAI-1 is thought to play an important role in the regulation of biological activity of tPA in blood. In our results, antigen levels of tPA in plasma both before and after the exercise did not show any significant differences between the two groups, but reduced fibrinolytic activities were observed after the exercise in the patient group despite significant differences in the mean exercise time, the mean work load and the mean RPP between the two groups. The main cause for this difference is probably due to decreased free tPA and increased PAI-1 levels and not due to an impaired release of tPA from vascular beds. There was no positive correlation between the plasma antigen levels of tPA and fibrinolytic activities, and the patient group showed normal tPA values and a trend toward a higher plasma antigen levels of tPA compared with controls. This hypothesis was also supported by the results from electrophoretic and immuno-blotting analysis that a decrease of free tPA in euglobulin fractions was associated with the low fibrinolytic activities. These results suggest that PAI-1 has a central role in relation to biological tPA activity in blood.

With respect to the importance of PAI-1 in the regulation of fibrinolytic activity in the blood, Lucore et al\(^ {22}\) reported that infused tPA in the thrombolytic therapy of acute myocardial in-
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fraction initially saturates PAI-1 and circulates as free tPA and in complexes with other protease inhibitors. In addition, that increased PAI-1 activity, associated with infarction, attenuates tPA activity after infusion even though immunoreactive tPA concentrations exceed pretreatment values. Therefore, we concluded that it was not enough to evaluate only plasma antigen levels of tPA but that it was necessary to evaluate fibrinolytic activities and tPA inhibitory activities simultaneously. Wiman et al. reported the method for detecting plasma tPA inhibitory activity, but by their method a standard curve could not always be plotted reproducibly (unpublished data). Therefore, we did not investigate plasma tPA inhibitory activity in the present study.

It is reported that a large part of PAI-1 exists in plasma in latent form and an active form is less than 10% of the total antigen levels of PAI-1 in plasma. Moreover, only active PAI-1 can make a complex with tPA and inactivate it. Sakata et al. reported that a storage pool on the surface of endothelial cells or the extracellular matrix produced by endothelial cells contains all the active PAI-1, and reaction between plasminogen activator and PAI-1 mainly occurs on the endothelial cell membranes in vitro studies. In the present study, decreased free tPA in the patients with lower fibrinolytic activities might be caused by increased inactivation of tPA on the impaired sclerotic endothelial surface in the patients, however the precise mechanism of the inactivation of tPA in vivo still remains unknown.

Although ELISA for antigen levels of PAI-1 in plasma has been developed and is available now, it is impossible to distinguish active PAI-1 from latent one using this method but use of a specific antibody against active or latent PAI-1 may resolve this problem in the near future.

Through these experimental analyses, it was concluded that reduced fibrinolytic activity observed in patients with CAD was considered to be due to decreased free tPA because of the complex formation of tPA with PAI-1 and that this might be one of the potential causes for the initiation and development of coronary artery disease.

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