Plasma plasmin–α2-plasmin inhibitor complex levels are increased in systemic sclerosis patients with pulmonary hypertension

M. Jinnin, H. Ihn, K. Yamane, Y. Asano, N. Yazawa and K. Tamaki

Objective. To determine the frequency and clinical significance of plasma plasmin–α2-plasmin inhibitor complex (PIC) in patients with systemic sclerosis (SSc).

Methods. Plasma samples from 74 patients with SSc and 32 healthy volunteers were examined by a specific enzyme-linked immunosorbent assay.

Results. Elevated plasma PIC levels were present in 35 of the 74 patients (47.3%) with SSc. The patients with elevated plasma PIC levels had pulmonary hypertension (PH) at a significantly higher incidence than those with normal PIC levels (31.4 vs 7.7%, \(P < 0.01\)). When PH was classified into isolated PH (IPH) and secondary PH (SPH), the presence of IPH was significantly greater in patients with elevated PIC levels than in those with normal levels (25.7 vs 5.1%, \(P < 0.02\)).

Conclusions. These results suggest that plasma PIC levels may be a marker of PH, especially IPH, in patients with SSc.

KEY WORDS: Fibrinolytic system, Microvascular obliteration, Enzyme-linked immunosorbent assay, Doppler echocardiography.

Scleroderma, or systemic sclerosis (SSc), is a generalized connective tissue disease which is characterized by microvascular obliteration and increased deposition of extracellular matrix such as collagen, resulting in fibrotic lesions [1, 2]. Pulmonary hypertension (PH) is a common occurrence in SSc, and severe PH leads to death [3]. PH associated with SSc can be due primarily to pulmonary vascular abnormalities or can be secondary to cardiac or interstitial lung involvement. The former is regarded as isolated pulmonary hypertension (IPH) and usually develops in patients with minimal or no pulmonary fibrosis [4]. In contrast, patients with PH due to severe pulmonary fibrosis are considered to have secondary PH (SPH). These two types of PH were classified into ‘pulmonary arterial hypertension related to collagen vascular disease’ and ‘pulmonary hypertension associated with interstitial lung disease’, respectively, as a result of the recent classification suggested in a World Health Organization symposium in 1998 [5].

Many clinical and laboratory features of SSc patients with PH have been reported to date. Isolated diffusion capacity for carbon monoxide (%DLco) reduction is an indicator for IPH [6]. The presence of other autoantibodies, such as anti-endothelial cell antibodies, has also been shown to be predictive for IPH [7]. Another study showed that the nitric oxide concentration in exhaled air is decreased [8]. We previously reported that an elevated erythrocyte sedimentation rate and increased IgG were common features of the SSc patients with PH, and SSc patients with PH tended to have pitting scars/ulcers [9]. Furthermore, we reported that β2-glycoprotein I-dependent anticardiolipin antibody, the presence of which might be associated with thrombosis, was significantly correlated with the presence of IPH in patients with SSc [10]. The presence of thrombosis activates the subsequent fibrinolysis, as seen in disseminated intravascular coagulation. We therefore determined the frequency and clinical significance of plasmin–α2-plasmin inhibitor complex (PIC), a fibrinolytic marker that directly reflects the generation of plasmin in vivo, in SSc patients.

Patients and methods

Clinical assessment

Plasma samples were obtained from 74 patients with SSc (seven men and 67 women; age range: 27–81 yr, mean: 59.2 yr) and 32 healthy volunteers. Plasma samples from 74 patients with SSc and 32 healthy volunteers were examined by a specific enzyme-linked immunosorbent assay. Elevated plasma PIC levels were present in 35 of the 74 patients (47.3%) with SSc. The patients with elevated plasma PIC levels had pulmonary hypertension (PH) at a significantly higher incidence than those with normal PIC levels (31.4 vs 7.7%, \(P < 0.01\)). When PH was classified into isolated PH (IPH) and secondary PH (SPH), the presence of IPH was significantly greater in patients with elevated PIC levels than in those with normal levels (25.7 vs 5.1%, \(P < 0.02\)).

Conclusions. These results suggest that plasma PIC levels may be a marker of PH, especially IPH, in patients with SSc.
healthy controls. All patients with SSC were grouped according to the classification system proposed by LeRoy et al. [11]: 27 patients had diffuse cutaneous SSC (dcSSC) and 47 patients had limited cutaneous SSC (lcSSC), as described previously [10]. All patients fulfilled the criteria proposed by the American College of Rheumatology [12]. Clinical and laboratory data reported in this study were obtained at the time of plasma sampling. Thirty-five of 74 patients had antiplatelet medications (riboflavin hydrochloride, beraprost sodium or sarpogrelate hydrochloride) and 25 patients had corticosteroid medications. Patients were evaluated for the presence of gastrointestinal, pulmonary, cardiac, renal or muscle involvement as described previously [10].

**Echocardiographic evaluation**

Echocardiographic studies were performed as described previously [9]. PH was defined as a right ventricular systolic pressure (RVSP) of more than 40 mmHg by Doppler echocardiography [13]. According to the results of echocardiography and the severity of pulmonary fibrosis (PF), the patients with PH were classified into two groups [4, 14]: patients with isolated PH (IPH; PH without severe PF), and those with secondary PH (SPH; PH with severe PF). Patients with severe pulmonary fibrosis, as seen on chest radiography and chest computed tomography, or patients having restrictive disease revealed by a pulmonary function test (vital capacity < 70% of predicted volume) were considered as having SPH. All remaining patients with PH were considered as having IPH.

**Antinuclear antibodies**

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence using HEP-2 cells as the substrate and double immunodiffusion, as described previously [15].

**Measurement of PIC concentrations**

Venous blood from all patients and control subjects was drawn into glass syringes containing 3.8% sodium citrate. Blood samples were centrifuged at 3000 rpm for 10 min, and plasma was drawn off and stored at −80°C until used. Aliquots of plasma were diluted 800-fold with saline and used to measure PIC concentrations, using a TD80C kit (Teijin, Tokyo, Japan) [16].

Plasma samples were incubated for 1 h at 37°C with peroxidase-labelled liquid-phase mouse monoclonal anti-2-plasmin inhibitor antibody and solid-phase rabbit polyclonal anti-plasminogen antibody (fixed on plastic beads 6 mm in diameter). After incubation the beads were washed twice with saline, transferred to new tubes, and further incubated for 30 min at 37°C with 400 μl of peroxidase-reactant substrate. The reaction was stopped by the addition of 0.25 M oxalic acid. The development of the colour was monitored at 420 nm with a TD80C photometric immunoassay and thrombin–antithrombin complex (by enzyme-linked immunosorbent assay, ELISA).

**Statistical analysis**

Statistical analysis was carried out with Student’s t-test for the comparison of means, and Fisher’s exact probability test for the analysis of frequency. Correlations with clinical data were assessed by Pearson’s correlation coefficient. P values less than 0.05 were considered significant.

**Results**

**Plasma concentrations of PIC**

The plasma concentrations of PIC were significantly higher in patients with SSC than in healthy controls (mean ± s.d.: 0.92 ± 0.39 vs. 0.41 ± 0.14 μg dl, P < 0.000001). As shown in Fig. 1, the mean plasma PIC concentration of patients with lcSSC was slightly elevated compared with the mean of patients with dcSSC (0.97 ± 0.41 vs. 0.83 ± 0.35 μg dl). When the cut-off value was set at 0.8 μg dl as mentioned above, elevated plasma concentrations of PIC were found in 23 of the 47 (49%) lcSSc patients and 12 of the 27 (44%) dcSSc patients. We also determined the plasma levels of several other factors associated with the coagulation–fibrinolysis system, such as plasminogen, antithrombin III, protein C, protein S, z2-plasmin inhibitor, D-dimer and thrombin–antithrombin complex, showing no differences between patients with SSC and healthy controls, and showing no correlation between these factors and the presence of PH. Moreover, we examined the plasma PIC levels in eight dermatomyositis patients with pulmonary fibrosis. Only one patient had elevated PIC levels and there was no significant difference between dermatomyositis patients and healthy controls in plasma PIC levels (data not shown).

**Correlation of PIC with clinical manifestations and laboratory data**

Table 1 shows the association of plasma PIC levels with the clinical and laboratory features in patients with SSC. There was no significant difference between these groups in terms of sex or mean age at onset. Patients...
TABLE 1. Correlation of plasmin–2-plasmin inhibitor complex (PIC) levels with clinical and serological features in patients with systemic sclerosis

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Patients with elevated PIC levels (n = 35)</th>
<th>Patients with normal PIC levels (n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male:female)</td>
<td>2.33</td>
<td>5.34</td>
</tr>
<tr>
<td>Mean age at onset (yr)</td>
<td>42.7</td>
<td>41.9</td>
</tr>
<tr>
<td>Type (diffuse:limited)</td>
<td>12:23</td>
<td>15:24</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitting scars/ulcers</td>
<td>51.4</td>
<td>46.2</td>
</tr>
<tr>
<td>Nailfold bleeding</td>
<td>69.7</td>
<td>63.2</td>
</tr>
<tr>
<td>Raynaud’s phenomenon</td>
<td>100</td>
<td>89.7</td>
</tr>
<tr>
<td>Telangiectasia</td>
<td>60.0</td>
<td>38.5</td>
</tr>
<tr>
<td>Calcinosis</td>
<td>11.4</td>
<td>5.1</td>
</tr>
<tr>
<td>Leg ulcer</td>
<td>8.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Sjögren symptoms</td>
<td>78.6</td>
<td>65.5</td>
</tr>
<tr>
<td>Organ involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>54.5</td>
<td>55.3</td>
</tr>
<tr>
<td>Decreased % VC</td>
<td>17.6</td>
<td>20.5</td>
</tr>
<tr>
<td>Decreased % DLeo</td>
<td>50.0</td>
<td>29.7</td>
</tr>
<tr>
<td>Pulmonary hypertension (PH)</td>
<td>31.4*</td>
<td>7.7</td>
</tr>
<tr>
<td>Isolated PH</td>
<td>25.7**</td>
<td>5.1</td>
</tr>
<tr>
<td>Secondary PH</td>
<td>5.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>55.6</td>
<td>73.3</td>
</tr>
<tr>
<td>Heart</td>
<td>8.6</td>
<td>7.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.9</td>
<td>10.3</td>
</tr>
<tr>
<td>ANA specificity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-topo I</td>
<td>25.7</td>
<td>15.4</td>
</tr>
<tr>
<td>Anti-centromere</td>
<td>40.0</td>
<td>25.7</td>
</tr>
<tr>
<td>Anti-U1 RNP</td>
<td>8.6</td>
<td>17.9</td>
</tr>
</tbody>
</table>

Unless indicated, values are percentages.

% DLeo, diffusion capacity for carbon monoxide; VC, vital capacity; ANA, antinuclear antibodies; Anti-topo I, anti-topoisomerase I antibodies; Anti-centromere, anticientromere antibodies; Anti-U1 RNP, anti-U1-ribonucleoprotein antibody; *P < 0.01, **P < 0.02 vs patients with normal PIC levels using Fisher’s probability test.

with elevated PIC levels had PH at a significantly higher incidence than those with normal PIC levels (31.4 vs 7.7%, P < 0.01). The SSc patients with PH had significantly higher concentrations of PIC than those without PH (1.33 ± 0.53 vs 0.83 ± 0.29 μg/dl, P < 0.00001). When PH was classified into IPH and SPH, the frequency of IPH was significantly greater in patients with elevated PIC levels than in those with normal levels (25.7 vs 5.1%, P < 0.02), whereas there was no correlation between PIC levels and SPH. Moreover, the plasma concentrations of PIC were correlated with RVSP (r = 0.529, P < 0.001). There was no significant difference in plasma PIC levels between patients with antiplatelet or corticosteroid medications and those without. A longitudinal study of 20 patients with increased plasma PIC levels showed few changes from before to after treatment with, for example, corticosteroid or antiplatelet medication.

Discussion

The putative active component of the process of fibrinolysis is plasmin. Plasma levels of plasmin are considered to reflect fibrinolytic activity and are therefore useful in detecting abnormalities in the fibrinolytic system. Because plasmin rapidly forms inactive complexes with its inhibitors such as 2-plasmin inhibitor and 2-macroglobulin in ratios of 1:1, the detection and quantification of PIC in plasma (which is more stable than plasmin in vivo) could serve as a sensitive probe for quantifying plasmin levels in plasma [16].

The activation of the fibrinolytic system generally occurs after the generation of fibrin thrombus in vivo. Therefore, the elevation of PIC levels can indicate the presence of thrombosis. High levels of PIC are observed in patients with disseminated intravascular coagulation [16], thromboembolic disease [18–20], hepatic failure [21] and malignancy [22]. Moreover, PIC is also elevated by fibrinolytic therapy with urokinase or tissue plasminogen activator [23]. On the other hand, PIC concentrations do not appear to be influenced by antiplatelet or corticosteroid therapy [24, 25]. In our study, neither antiplatelet medications, which are usually used for PH as the preventative therapy against secondary thrombosis, nor corticosteroid medications affected PIC concentrations significantly.

As for collagen diseases, Kawakami et al. [16] reported that concentrations of PIC were elevated in 75% of patients with systemic lupus erythematosus (SLE) and 82% of patients with rheumatoid arthritis (RA). In their study, plasma PIC levels were found to be 1.9 ± 1.4 μg/dl in SLE patients, 1.6 ± 0.6 μg/dl in RA patients and 0.4 ± 0.2 μg/dl in healthy subjects, results which are compatible with our own. They also speculated that PIC may be useful for detecting and evaluating the severity and the activity of vasculitis in patients with these diseases [16], and that PIC levels are elevated in patients with active SLE [26]. It has been reported that PIC levels were increased in three of eight patients (37.5%) with SSc [25], which is similar to our result (47.3%). In our study, patients with high levels of PIC had PH, especially IPH, at a significantly higher prevalence. Defects of anticoagulant factors such as antithrombin III, protein C and protein S, or the local increase of plasminogen activator inhibitor-1 (PAI-1), which inhibits the activity of tissue plasminogen activator, have been suggested as the pathogenesis of thrombosis in PH [27–30]. We speculate that high levels of PIC in SSc patients with PH may result from generation of thrombus, which may cause PH.

However, we found no abnormality in the levels of several other factors associated with the coagulation–fibrinolysis system, such as plasminogen, antithrombin III, protein C, protein S, 2-plasmin inhibitor, D-dimer or thrombin–antithrombin complex. In addition, there was no correlation between these factors and the presence of PH. Taking into account these normal results, there is some possibility that PIC is merely an acute-phase reactant of active disease. But a more specific basis for this discrepancy between plasma PIC levels and the levels of other factors may be that chronically inflamed tissues release large amounts of plasminogen activator, leading to accelerated plasmin production and hence elevated levels of PIC in vivo [16, 31].
Doppler echocardiography as employed in our study is reported to be a reliable method for detecting PH, no less than right heart catheterization [32], but there is some possibility that we missed cases of PH. Furthermore, in the longitudinal study of 20 patients, there were hardly any changes of plasma PIC levels. Thus, further studies are needed to examine whether PIC is a parameter of PH severity or activity.

In conclusion, our study suggested that plasma PIC levels are a marker of PH, especially IPH, in patients with SSc. Although the coagulation–fibrinolysis system may play a role in the pathogenesis of PH, our normal results for other parameters of coagulation and fibrinolysis suggest that elevation of PIC may stem from a more isolated release of plasminogen.

References