Normal Titer of Functional and Immunoreactive Protein-C Inhibitor in Plasma of Patients with Congenital Combined Deficiency of Factor V and Factor VIII

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Protein-C inhibitor (PCI) is a newly described plasma inhibitor directed against a vitamin-K-dependent serine protease, activated protein-C, which is involved in the inactivation of factor V and factor VIII. Marlar and Griffin have reported that PCI activity is absent in the plasma of patients with congenital combined factor V/VIII deficiency. We have measured the levels of PCI in the plasma of seven unrelated patients with this disorder using both functional and immunologic methods. The rate at which the amido lytic activity of activated protein-C was neutralized in the patients' plasma was essentially identical to that observed in normal plasma. The titer of PCI antigen, as measured by an electroimmunoassay using a monospecific anti-PCI serum, was $5.3 \pm 1.6 \mu g/ml$ in the patients' plasma and was not significantly different from that of normal plasma ($5.3 \pm 2.7 \mu g/ml, n = 30$). The levels of factor-V-related antigen, factor V coagulant antigen, and factor VIII coagulant antigen were low in all patients' plasma and were in good agreement with their respective coagulant activity. Our results do not appear to support the hypothesis that combined factor V/VIII defect is due to a lack of PCI.

However, they have found considerable variations in PCI titer among different patients. Some patients' plasma contained very low PCI activity, while others demonstrated normal activity.

We have recently isolated and characterized PCI from human plasma and have studied the mechanisms of action upon activated protein-C. In the present study we prepared a monospecific antiserum against PCI and measured the level of PCI in the plasma of seven unrelated patients with combined factor V/VIII deficiency, using both functional and immunologic methods. Factor V and factor VIII coagulant antigen were also studied in these plasmas.

MATERIALS AND METHODS

All chemicals from commercial sources were the best grade available. Bovine serum albumin (RIA grade) and heparin were purchased from Sigma Chemical Co., St. Louis, MO. The synthetic substrates, 7-butyloxycarbonyl-Leu-Ser-Thr-Arg-4-methylcoumarylaldehyde (peptide-MCA) were obtained from Peptide Institute Inc., Osaka, Japan. Factor-V-deficient and factor-VIII-deficient plasma were purchased from George King Biochemicals, Overland, CA. Rabbit brain thromboplastin (Simplastin) and rabbit brain cephalin (Actin) were obtained from Warner-Lambert Co., Morris Plains, NJ and Dade Diagnostic Inc., Miami, FL, respectively. Freegen was obtained from Hoechst Behringer, Mannheim, W. Germany.

Plasma

Plasma samples from normal individuals and patients deficient in factor V, factor VIII, or combined factor V/VIII were prepared with 9 parts whole blood (obtained by venipuncture) and 1 part 3.8% trisodium citrate in polyethylene tubes, and centrifuged at 3,000 g for 10 min at 4°C. Citrated plasma from each patient was frozen at $-70^\circ$C until used. The plasma pool from 30 normal individuals was prepared as described and used as a standard plasma. Some plasma samples from patients diagnosed as combined factor V/VIII deficiency were kindly provided by Drs. O. D. Ratnoff (patient 1), F. Kuto (patient 2), and A. Teraoka (patient 7). Other combined factor-V/VIII-deficient plasmas were from patients of the Hematology Clinic at Nagoya University Hospital. One of these patients has been reported previously.
Proteins

PCI and activated protein-C were purified and prepared as described earlier.10,17 Human factor V was purified according to a previously described method.18

Antiserum

Antisera against purified PCI and factor V were raised by immunizing rabbits with respective purified proteins in Freund’s complete adjuvant. Both antisera formed a single precipitin arc against the respective purified proteins upon immunoelectrophoresis. Anti-PCI rabbit immunoglobulin isolated from the serum removed all of the plasma inhibitory activity against activated protein-C, as described.17

Determination of PCI Activity

The activity of PCI in plasma was measured as follows. First, 50 μl of activated protein-C (10 μg/ml) in buffer, consisting of 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, 2 mM CaCl2, 0.1% bovine serum albumin with or without heparin (5 U/ml final), and 50 μl of plasma sample diluted in the same buffer were mixed and incubated at 37°C in polystyrene tubes for 15 min. Twenty-microliter aliquots were then withdrawn and added to 2.0 ml of 100 μM peptide-MCA, dissolved in 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 2 mM CaCl2, and kept at 37°C. Changes in the intensity of the fluorescence of 7-amino-4-methycoumarin (AMC) liberated from the substrate were then measured at 440 nm by an initial-rate method, as described.8 The activity of activated protein-C was expressed as percent in comparison with a standard normal plasma. A sample diluted in the same buffer were mixed and incubated at 37°C, was expressed as micrograms per milliliter plasma.

Determination of PCI Antigen (Immunoreactive PCI)

The concentration of PCI in plasma was determined by an electroimmunoassay using a monospecific antisera.20 After electrophoresis of plasma samples (20 μl/well) on 1% agarose gels (2-mm thickness) containing 1% anti-PCI serum, the gel was washed with Tris-saline overnight to remove unprecipitated proteins. Thereafter, the gel was dried, stained with 0.5% Coomassie brilliant blue in 50% methanol and 10% acetic acid, and destained in 10% methanol and 10% acetic acid. Serial dilutions of purified PCI were included in each plate and used as a standard. The results were expressed as micrograms per milliliter plasma.

Factor V Assay Methods

The coagulant activity of factor V was estimated by one-stage prothrombin time using Simplastin and congenital factor-V-deficient plasma, as previously described.18

The concentration of factor-V-related antigen (V:RAg) was measured by Laser nephrometric assay using a monospecific rabbit anti-human factor V serum (Laser nephrometer, Hoechst Behringer, Mannheim, W. Germany). Two hundred microliters of 1/20 dilution of antiserum and 100 μl of normal or patient’s plasma, which were previously treated with Freegen as noted in the manufacturer’s instructions, were mixed in a cell cuvette at room temperature overnight, and the immunoprecipitate was then measured according to the manufacturer’s instructions. The results were expressed as percent V:RAg in comparison to normal pooled plasma, which was arbitrarily defined as containing 100%.

Factor VIII (AHF) Assay Methods

The coagulant activity of factor VIII was measured by an activated partial thromboplastin time method, using Actin and congenital factor-VIII-deficient plasma.21 Factor-VIII-related antigen (VIII:RAg) was determined by a single radial immunodiffusion test using L-partigen plate (Hoechst, Behringer Mannheim, W. Germany) according to the manufacturer’s instructions.

Factor VIII coagulant antigen (VIII:CAg) was estimated by antibody neutralization, using a human circulating anticoagulant against factor VIII that had been developed in a patient with classic hemophilia.22 The results were expressed as percent VIII:CAg in comparison to normal pooled plasma, which was arbitrarily defined as containing 100%.

RESULTS

Inhibitory Activity Against Activated Protein-C in Normal and Combined Factor-V/VIII-Deficient Plasma

Figure 1 shows the time-dependent inhibition of plasma from normal individuals and patients with the combined defect against activated protein-C. As reported previously,9,10 normal human plasma inactivated the activity of activated protein-C progressively. Plasma of all seven patients with the combined deficiency blocked the activated protein-C activity at the same rate as normal plasma.

It has been shown that the rate of the reaction between PCI and activated protein-C is accelerated by heparin at an optimal concentration (5 U/ml) to about 30-fold, compared with that in the absence of heparin.12 Thus, the inhibition of activated protein-C by normal and combined deficiency plasma was also examined in the presence of heparin (Fig. 2). There was no difference between normal and combined factor-V/VIII-deficient plasma in the rate of the inactivation of activated protein-C under these conditions.

PCI Level in Normal and Combined Factor-V/VIII-Deficient Plasmas

Figure 3 shows the titer of plasma PCI as determined by the inhibition of amidolytic activity of activated protein-C (PCI activity) and by electroimmunoassay (PCI antigen). PCI activity in the plasma of patients was within the range of normal individuals. PCI antigen in the patients’ plasma was 5.3 ± 1.6 μg/ml and was not different from that of normal plasma (5.3 ± 2.7 μg/ml). Both PCI activity and antigen were normal in the plasma of 3 patients with classical hemophilia and 3 patients with hereditary
factor V deficiency. Figure 4 shows the typical patterns of electroimmunoassay of plasma PCI antigen.

**Factor V and Factor VIII Coagulant and Related Antigen**

As shown in Table 1, both V:RAg and V:CAg levels in combined factor-V/VIII-deficient plasma were low in comparison with the coagulant factor V activity. VIII:RAg was normal in all patient plasma. In contrast, VIII:CAg was deficient in these plasma samples in comparison with the coagulant factor VIII activity.

Both the concentrations of V:RAg and V:CAg were markedly low (less than 2% of normal) in plasmas of 3 patients with factor V deficiency. The levels of VIII:RAg in plasmas of 3 patients with classical

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**Fig. 1.** Inactivation of activated protein C by pooled normal and combined factor-V/VIII-deficient plasma. Activated protein-C was incubated with plasma and aliquots of the incubation mixtures were removed at intervals to determine the residual activity of activated protein-C, as noted in Materials and Methods. Numbers from 1 to 7 correspond to the respective patients' plasma as indicated in Table 1. Eight and 9 correspond to normal pooled plasma and the buffer as a control, respectively.

**Fig. 2.** Inactivation of activated protein-C by pooled normal and combined factor-V/VIII-deficient plasma in the presence of heparin. At 5 min (arrow) after incubation of activated protein-C with plasma, heparin (5 U/ml final) was added. Assay method and the numbers of samples were the same as indicated in Fig. 1.

**Fig. 3.** Concentrations of plasma PCI determined by bioassay and electroimmunoassay. The mean plasma PCI activities of normal individuals (n = 30) and combined deficient patients (n = 7) were 105.3% ± 36.0% and 93.3% ± 18.6%, respectively, when normal pooled plasma was defined arbitrarily as containing 100% PCI activity. The mean plasma PCI antigen of normal individuals and combined deficient patients were 5.3 ± 2.7 µg/ml and 5.3 ± 1.6 µg/ml, respectively.
hemophilia were normal, whereas VIII:CAg levels ranged from 2% to 15% of normal and paralleled those of the coagulant factor VIII activity.

**DISCUSSION**

The PCI, first described by Marlar and Griffin, has drawn attention as a new inhibitor that is present in normal plasma but absent in congenital combined factor-V/VIII-deficient plasmas. We have recently isolated and characterized PCI that retained biologic activity. PCI consists of a single polypeptide chain with an apparent molecular weight of 57,000. It is different chemically and immunologically from other known plasma protease inhibitors, including the recently found heparin cofactor II. The purified inhibitor hampers the amidolytic and proteolytic activity of activated protein-C by forming an apparent complex with the enzyme. Interestingly, the reaction rate between PCI and activated protein-C is accelerated by relatively higher concentrations of heparin and dextran sulfate. Similar evidence was obtained by Canfield and Kisiel. Thus, PCI might belong to a category of heparin cofactors in plasma.

The present study aimed at examining whether PCI is truly absent in plasma of the patients with combined factor V/VIII deficiency. All seven patients' plasmas inhibited the activity of activated protein-C at the same rate as normal plasma, as measured in an amidolytic assay. Moreover, the rate of this reaction was accelerated by the addition of heparin to the same degree as in normal plasma. Furthermore, the levels of PCI antigen (immunoreactive PCI), as estimated using a monospecific anti-PCI serum, were within the normal range in these patients. These data are at variance with those of Marlar and Griffin and do not support the hypothesis, now accepted in general, that a congenital lack of PCI forms the molecular basis of the combined factor V/VIII deficiency. Giddings et al. reported that there were less severely reduced levels of PCI activity in 21 patients with this disorder. It is noteworthy that some of their patients' plasma contained normal PCI titer, while others showed no PCI activity. Whether or not these conflicting results reflect the heterogeneous nature of the combined defect is not clear at present.

After our study was completed, Canfield and Kisiel reported normal functional levels of PCI in four patients. They also demonstrated that the plasma

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**Table 1. The Levels of Factor V, Factor VIII, and Protein-C Inhibitor in Plasma From Patients With Combined Deficiency of Factor V/VIII**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Factor V (%)</th>
<th>Factor VIII (%)</th>
<th>Protein C Inhibitor</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>V/C V:CAg V:RAg</td>
<td>VIII:C VIII:CAg VIII:RAg</td>
<td>Activity (%)</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>4 11 7</td>
<td>4 3 95</td>
<td>80</td>
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<td>F</td>
<td>6 6 10</td>
<td>5 6 84</td>
<td>88</td>
</tr>
<tr>
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<td>M</td>
<td>12 12 13</td>
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</tr>
<tr>
<td>7</td>
<td>F</td>
<td>12 10 13</td>
<td>18 17 113</td>
<td>92</td>
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<tr>
<td>Pooled normal plasma*</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
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</table>

*Pooled plasma was a mixture of citrated plasma from 30 normal donors.
concentration of activated protein-C binding protein was normal in their patients as determined by an immunoassay. Whether or not our PCI and activated protein-C binding protein is the identical protein is not known. However, it is pertinent to point out that our purified PCI has the capacity to inhibit activated protein-C, whereas their activated protein-C binding protein has no apparent inhibitory activity, and also, that the plasma concentration of their protein is significantly higher than that of our PCI.

Since the balance between activated protein-C and PCI may influence the properties of factor V and factor VIII, we have also studied plasma V:RAg, V:CAg, VIII:RAg, and VIII:CAg in our patients. All seven patients had low levels of V:RAg, V:CAg, and VIII:CAg that paralleled their respective coagulant activities. Our findings are consistent with those of Canfield and Kisiel.25 In contrast, Giddings et al.10 found normal levels of V:CAg (as measured by inhibitor neutralization with a rabbit antibody) and VIII:CAg (as measured by inhibitor neutralization with a human antibody) in 7 of 21 patients studied. Whether these 7 patients belong to a unique subset of this disorder remains to be established. It is interesting to note that the level of PCI did not correlate significantly with those of V:CAg or VIII:CAg.10 The levels of VIII:RAg were normal in all of our 7 patients, which is consistent with previous reports.14,27

REFERENCES

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