CONCISE REPORT

Identification of Monoclonal Antibodies That Inhibit the Function of Protein C Inhibitor. Evidence for Heparin-Independent Inhibition of Activated Protein C in Plasma

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Monoclonal antibodies specific for protein C inhibitor (PCI) partially blocked the inactivation of activated protein C (APC) in plasma, whereas in a purified system, the PCI activity could be completely blocked. The inactivation of APC in normal and in PCI-depleted plasma was similar in the absence of heparin. The addition of heparin did not change the rate of inactivation of APC in PCI-depleted plasma, whereas in normal plasma a rapid phase of inhibition of APC was followed by a slower phase of inhibition.

PROTEIN C INHIBITOR (PCI) is a recently discovered inhibitor for activated protein C (APC). Suzuki et al.1 have purified PCI from human plasma. It is a single-chain glycoprotein with a molecular weight of 57,000. PCI forms a 1:1 molar complex with APC. At the same time, a small peptide at the carboxyl terminus is cleaved from PCI, which results in a modified, inactive PCI.2 PCI inactivates not only APC but also thrombin, factor Xa,3 kallikrein, and factor Xla.4 Furthermore, PCI is functionally and immuno logically related to the urokinase inhibitor present in urine.5,6 The cDNA for human PCI has been characterized, and the amino acid sequence of PCI is highly homologous with members of the superfamily of serine protease inhibitors, the serpins.7 In this study, we describe the characterization of monoclonal antibodies specific for PCI. Two monoclonal antibodies inhibit the function of PCI completely in a purified system but only partially in plasma. By using these monoclonal antibodies, we demonstrated the presence of a heparin-independent inhibitor of APC in plasma.

MATERIALS AND METHODS

Purification of proteins. Human PCI was purified as previously described.4 Human protein C was purified and activated as described by Koedam et al.1 Protein concentrations, except for PCI and purified antibodies, were determined by the method of Lowry et al.8 The concentration of PCI was determined with E1* (280 nm) = 14.1,1 and the concentration of purified antibodies was determined with E1* (280 nm) = 14.3.10

Preparation of monoclonal antibodies. Monoclonal antibodies were obtained by immunizing a BALB/c mouse with purified PCI as previously described.4 Seven clones (API-20, -25, -29, -30, -39, -88, and -104) specific for PCI were cultured in bulk by injection of hybridoma cells in pristane-primed mice. Monoclonal antibodies were purified from ascites fluid by protein A-Sepharose chromatography according to the instructions of the manufacturer (Pharmacia Laboratories, Uppsala, Sweden). The seven clones were of the IgG1 subtype.

Preparation of PCI-depleted plasma. PCI-depleted plasma was obtained by applying normal citrated plasma to a column of CNBr-activated Sepharose (Pharmacia) to which 20 mg of API-39 antibody (50% ammonium sulphate precipitation of ascites fluid) per gram of Sepharose was coupled according to the instructions of the manufacturer. The undiluted flow-through fractions were tested for PCI antigen with an enzyme-linked immunosorbent assay11 and were found to be totally depleted of PCI (<0.1%). Normal plasma consisted of a pool of plasma from 40 healthy volunteers. The plasmas were subsampled, snap-frozen, and stored at -70°C.

Characterization of monoclonal antibodies. Purified monoclonal antibodies (10 μL, 1 mg/mL) were incubated with 90 μL plasma for 30 minutes at 37°C. Subsequently, the remaining PCI activity was determined by the addition of APC (5 μL, 28 μg/mL) and heparin (5 μL, 50 U/mL) to 50 μL of the monoclonal antibody-plasma mixture. After 60 minutes, the APC amidolytic activity was determined by adding 45 μL of the mixture to 475 μL of 0.4 mmol/L S-2366 (KabiVitrum, Stockholm). A standard curve was constructed from dilutions of normal plasma.

In a purified system, 0.25 U/mL PCI in Tris buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% wt/vol bovine serum albumin) was incubated with different concentrations of monoclonal antibodies API-30 and -39 for 30 minutes at 37°C. The remaining PCI activity was determined as described earlier. A standard curve was constructed by using dilutions of purified PCI.

Inhibition of APC in plasma. The inhibition of APC in normal and PCI-depleted plasma was investigated by following the amidolytic activity of APC. Two hundred fifty microliters of normal or PCI-depleted plasma in the absence or presence of heparin (2 μg/mL) at 37°C. After the addition of APC (40 μg/mL, 25 μL), the amidolytic activity was followed by adding samples (35 μL) of the reaction mixture to 0.4 mmol/L S-2366 (465 μL) in 50 mmol/L Tris, pH 7.8, and 150 mmol/L NaCl.

The incubation of plasma with monoclonal antibody API-39 was performed by adding 25 μL of antibody (1 mg/mL) to 250 μL of plasma. After incubation for 30 minutes at 37°C, APC was added, and the amidolytic activity was determined as described earlier.

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Monoclonal antibodies against PCI were obtained by immunizing mice with purified PCI. The monoclonal antibodies were characterized by incubation of the antibodies with plasma, after which the remaining APC-inhibiting activity was measured with a functional assay. Two of the seven monoclonal antibodies (API-30, -39) reduced the level of APC-inhibiting activity in plasma to approximately 60% (Fig 1). On the other hand, incubation of monoclonal antibodies API-30 and -39 with purified PCI blocked the APC-inhibiting activity completely (Fig 2). This suggests the presence of a second inhibitor of APC in plasma, as previously mentioned by van der Meer et al.\textsuperscript{12} and Heeb et al.\textsuperscript{5} To study this phenomenon further, the inhibition of APC was investigated in normal and PCI-depleted plasma. Figure 3A shows the inhibition of APC in normal plasma in the presence or absence of heparin. In the presence of heparin, a biphasic inactivation of APC is observed; a fast first phase is followed by a slower second phase. In the absence of heparin, only the slower inactivation is observed. Subtracting the heparin-independent inhibition from the inhibition in the presence of heparin shows the heparin-dependent inactivation of APC, which is characterized by a rapid inactivation in ten minutes, after which no further inhibition is observed (Fig 3A). This may be caused by a complete consumption of PCI; however, based on the concentration of APC added and a 1:1 molar complex, this would lead to a lower PCI...
concentration (1.6 μg/mL) than has been obtained from specific activities of purified PCI (5 μg/mL). Alternative explanations may be that APC is protected from inactivation by PCI by a plasma factor or that APC inactivates PCI without the formation of a 1:1 enzyme-inhibitor complex.

The inactivation of APC in PCI-depleted plasma in the presence of heparin is similar to the inactivation in the absence of heparin (Fig 3B), thus indicating that PCI is the only heparin-dependent inhibitor of APC in plasma. Furthermore, it confirms the observation that PCI is a poor inhibitor of APC in the absence of heparin. Our results indicate the presence of an unknown inhibitor (or inhibitors) for PCI as previously suggested. The inhibition in PCI-depleted plasma is slower than the heparin-independent inhibition in normal plasma due to a partial loss of the heparin-independent inhibitor during the PCI-depletion of plasma. The addition of purified PCI to the PCI-depleted plasma restores the heparin-dependent inhibition of the plasma (Fig 3C).

Incubation of normal plasma with monoclonal antibody API-39 results in the loss of heparin-dependent APC inactivation (Fig 3D). Incubation with a control monoclonal antibody specific for protein S did not change the inactivation of APC in plasma (data not shown). This indicates, in agreement with the observation in a purified system (Fig 2), that monoclonal antibody API-39 is able to block the functional activity of PCI in plasma. The inhibition of APC in the presence of this monoclonal antibody is identical to the inactivation of APC in normal plasma without heparin, which indicates that PCI does not contribute to the heparin-independent inhibition of APC in plasma. This suggests that PCI is unable to inhibit APC in plasma without heparin. A physiologic role for PCI in vivo cannot be excluded since the vessel wall can participate in the inactivation reaction by providing heparinlike molecules. Furthermore, PCI has been recognized as a potent inhibitor for kallikrein and factor XIa in the absence of heparin, thereby forming a link between the contact system and the protein C pathway. Recent studies have shown that PCI is identical to plasminogen activator inhibitor 3, thus indicating that PCI is also involved in the regulation of the fibrinolytic system.

In conclusion, we have described two monoclonal antibodies (API-30, -39) that inhibit the functional activity of PCI. From these monoclonal antibodies evidence was obtained for heparin-independent inhibition of APC in plasma, without the participation of PCI in this inhibition.

REFERENCES
Identification of monoclonal antibodies that inhibit the function of protein C inhibitor. Evidence for heparin-independent inhibition of activated protein C in plasma

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