Anticoagulant Activity of Immobilized Thrombomodulin

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Bovine lung thrombomodulin was partially purified, and immobilized on agarose gel (Sepharose 4B). Immobilized thrombomodulin inhibited the procoagulant activity of thrombin, and enhanced the thrombin-catalyzed protein C activation. The plasma recalcification time test showed that immobilized thrombomodulin prolonged plasma clotting time. It is suggested that the immobilization of thrombomodulin will provide an antithrombogenic biomaterial able to convert thrombin from a procoagulant to an anticoagulant enzyme.

Keywords immobilized enzyme; thrombomodulin; protein C; thrombin inhibitor; antithrombogenic material

Introduction

In order to provide antithrombogenic biomaterials, the immobilization of anticoagulant or thrombolytic enzymes on biomaterials has been studied extensively by many investigators. For example, there have been reports on the immobilization of heparin on artificial polymer surfaces.[1-4] Although immobilized heparin inhibits surface-induced coagulation, the overall blood compatibility of these surfaces *in vivo* remains in question. We have reported previously that heparin immobilized on an agarose matrix bound activated coagulation factor X and thrombin, but could not inhibit these factors.[5] In addition, heparin has been shown to induce platelet aggregation in citrated platelet rich plasma and to enhance platelet aggregation and serotonin secretion induced by exogenous agents.[6]

It has been reported that procoagulant activities of thrombin are inhibited when thrombin binds to thrombomodulin, the endothelial cell membrane protein.[7] Thrombomodulin forms a noncovalent complex with thrombin. In this complex, thrombin fails to react with its natural substrates including fibrinogen, factor V, and platelets. However, the ability of thrombin to activate protein C is enhanced 1000 times by thrombomodulin[8] in the presence of Ca^{2+}. Activated protein C attenuates further thrombin generation by inactivating factor Va and VIIIa.[9] As a result of changes in the substrate specificity of thrombin caused by binding to thrombomodulin, thrombin changes from a procoagulant enzyme into an anticoagulant enzyme.

It is considered that thrombomodulin maintains the antithrombogenicity of the native blood vessel, along with other factors, such as prostacyclin (PGI_2) synthase.[10] Therefore, it is expected that the immobilization of thrombomodulin would improve the antithrombogenicity of artificial polymers. In this work, the anticoagulant activity of thrombomodulin-immobilized material was investigated.

Materials and Methods

Materials Bovine thrombin,[11] protein C,[12] and antithrombin III[13] were purified by published methods. Bovine fibrinogen, porcine mucosal heparin, and bovine serum albumin were purchased from Sigma. Tresyl-activated Sepharose 4B was purchased from Pharmacia. Lubrol PX, Triton Xp100, *tert*-butyloxycarbonyl-Leu–Ser–Thr–Arg–4-methylcoumaryl-7-amide (Boc–Leu–Ser–Thr–Arg–MCA), and 7-amino-4-methylcoumarin (AMC) were purchased from Nakarai Chemicals, Ltd. [^{14}C]-Serotonin was purchased from New England Nuclear.

Bovine lung thrombomodulin was purified according to the method developed for rabbit lung thrombomodulin.[8]

Assay of Thrombomodulin The assay mixture, which contained 10 μl each of protein C (200 μg/ml), thrombin (400 NIH unit/ml), 0.02 M Tris–HCl (pH 7.5) containing 0.1 M NaCl and 3.5 mM $CaCl_2$, and thrombomodulin sample, was incubated for 10 min at 37 °C. The reaction was terminated by the addition of 10 μl of antithrombin III (50 μg/ml) containing heparin (100 U/ml). Then 500 μl of 100 mM Boc–Leu–Ser–Thr–Arg–MCA solution was added to the reaction mixture, and incubation was carried out for 10 min at 37 °C. The reaction was terminated by the addition of 1 ml of 15% acetic acid. The liberated AMC was measured as described previously.[5] One unit of thrombomodulin is defined as the amount necessary to yield activated protein C which forms 1 nmol of AMC/min.

Preparation of Immobilized Proteins About 500 units of thrombomodulin or bovine serum albumin was added to 650 mg of Tresyl-activated Sepharose 4B suspended in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl. The reaction was continued for 4 h at room temperature. The gel was washed with 0.1 M $NaHCO_3$ containing 0.5 M NaCl, and with 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl and then resuspended in 0.01 M Tris–HCl buffer, pH 7.4, containing 0.145 M NaCl and stored at 4 °C.

Measurement of Fibrin Clotting Immobilized thrombomodulin suspension (100 μl) was added to 100 μl of 1% fibrinogen solution, and the mixture was incubated for 3 min at 37 °C. One hundred microliter of thrombin was added to the mixture to give a final concentration of 0.17 unit/ml, and clotting time was measured by using a Fibrometer (Becton, Dickinson and Co.).

Preparation of Blood Sample For the aggregation study, rabbit platelet-rich plasma (PRP) was prepared by centrifugation of citrated (0.38%, final concentration) blood at $150 \times g$ for 7 min at room temperature. The remaining blood sample was centrifuged again at $2300 \times g$ for 15 min to prepare platelet poor plasma (PPP), and then PRP was diluted with PPP to give a final concentration of about 10^6 platelets/μl. The concentration of platelets was measured with a platelet counter (Toa Iyodensi, PL110).

For platelet release reaction study, ethylenediaminetetraacetic acid (EDTA) tripotassium salt was used as an anticoagulant, and PRP was prepared by the method described above. Washed platelets were prepared from the PRP by the method of Tollefsen *et al.*,[14] and suspended to give a final concentration of about 4×10^5 platelets/μl.

For the plasma recalcification time test, bovine PPP was prepared by centrifugation of citrated blood (obtained from a slaughterhouse) at $8000 \times g$ for 15 min at 4 °C, and was stored at −80 °C until used.

Measurement of Platelet Aggregation Platelet aggregation was measured by the turbidimetric method using platelet aggregometer (Rika Denki). Inhibition of platelet aggregation was measured by the addition of samples to 250 μl of PRP prior to the addition of thrombin as a platelet aggregating agent. Thrombin was added to give the final concentration of 0.3 unit/ml.

Measurement of Platelet Serotonin Release Release of [^{14}C]serotonin was measured by the method of David and Herion.[15] Thrombin was added to give the final concentration of 0.2 unit/ml. Release of [^{14}C]Serotonin was measured in the absence of immobilized thrombomodulin. Complete inhibition of the release was defined as 100% inhibition. Inhibition of serotonin release was measured by the addition of samples to 250 μl of PRP prior to the addition of thrombin.

Plasma Recalcification Time Test Immobilized thrombomodulin suspension (100 μl) containing 14 mg of gel was added to 100 μl of citrated bovine plasma, and then incubated for 3 min at 37 °C. One hundred

microliters of 25 mM $CaCl_2$ was added to the mixture, and clotting time was measured with the Fibrometer.

Results

Bovine lung thrombomodulin was purified 1920-fold over Triton X-100-solubilized membrane fraction. The specific activity was 19800 units/mg protein, and the activity yield was 11%. This purified thrombomodulin was used for immobilization experiments. The specific activity of immobilized thrombomodulin prepared by the tresyl-activation method was 0.31 unit/mg dry gel, and the activity yield was 40%.

Figure 1 shows the influence of immobilized thrombomodulin on thrombin's ability to clot fibrinogen. Immobilized albumin (47 mg dry gel/ml) did not affect the clotting time. In contrast, immobilized thrombomodulin (10 mg dry gel/ml) prolonged the clotting time. As the amount of immobilized thrombomodulin increased, there was progressive increase in the clotting time followed by a upward break in the curve. Immobilized thrombomodulin at 47 mg dry gel/ml prolonged the clotting time to about 700 s, in contrast to 75 s for the same amount of immobilized albumin.

Figure 2 shows the influence of immobilized thrombomodulin on thrombin-induced platelet aggregation. The thrombin was added at a concentration which did not cause fibrin clot formation before the maximal platelet aggregation was reached. Immobilized albumin had no effect on thrombin-induced platelet aggregation. Thrombin-induced aggregation was inhibited by the addition of immobilized thrombomodulin in a dose-dependent manner. Seventy-five percent inhibition was produced by the addition of 42 mg dry gel/ml of immobilized thrombomodulin.

The influence of immobilized thrombomodulin on thrombin-induced serotonin release of platelet was studied. Figure 3 shows that immobilized thrombomodulin inhibited serotonin release in a dose-dependent manner. Immobilized thrombomodulin at 42 mg dry gel/ml achieved approximately 100% inhibition.

Immobilized thrombomodulin stimulated the thrombin-catalyzed activation of protein C as shown in Fig. 4. When the amount of thrombin was held constant, the protein C activation depended upon the amount of immobilized thrombomodulin.

The anticoagulant activity of immobilized thrombomodulin was evaluated by use of the plasma recalcification time test. Table I shows that immobilized thrombomodulin clearly prolonged clotting time compared with immobilized albumin.

The immobilized thrombomodulin was incubated in citrated plasma at 37 °C for 150 min. After washing with 0.1 M $NaHCO_3$ containing 0.5 M NaCl, then with 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl, the activity

TABLE I. Effect of Immobilized Thrombomodulin on Plasma Clotting Time

Material	Clotting time (s)[a]
Immobilized bovine serum albumin	323.5±26.9 (n=5)
Immobilized thrombomodulin	450.4±52.3 (n=6)[b]

a) Values are means±S.D. *b*) $p<0.01$.

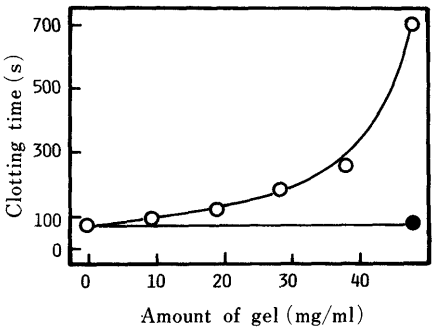

Fig. 1. Inhibition of Thrombin-Catalyzed Fibrin Formation by Immobilized Thrombomodulin

○, immobilized thrombomodulin; ●, immobilized albumin.

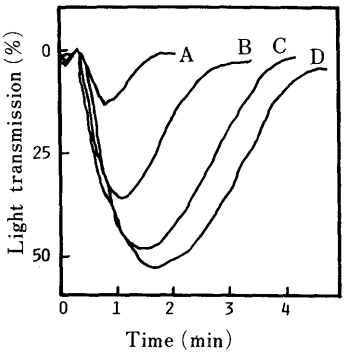

Fig. 2. Inhibition of Thrombin-Induced Platelet Aggregation by Immobilized Thrombomodulin

Immobilized thrombomodulin (A, 42 mg dry gel/ml; B, 21 mg dry gel/ml; and C, 8.5 mg dry gel/ml) and immobilized albumin (D, 42 mg dry gel/ml) were added to PRP prior to the addition of thrombin.

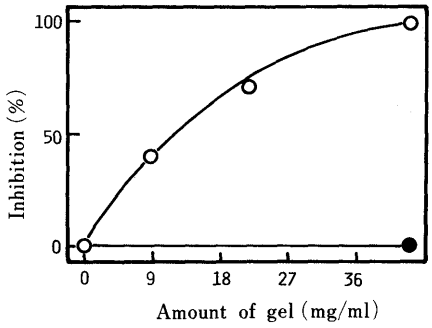

Fig. 3. Inhibition of Thrombin-Induced Platelet Serotonin Release by Immobilized Thrombomodulin

○, immobilized thrombomodulin; ●, immobilized albumin.

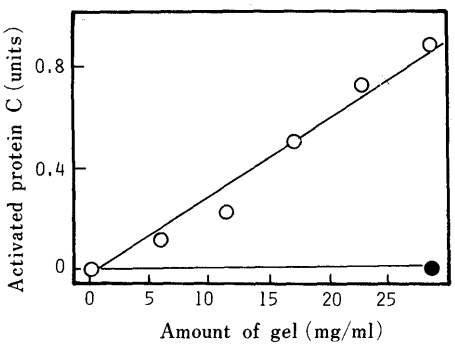

Fig. 4. Acceleration of Thrombin-Catalyzed Protein C Activation by Immobilized Thrombomodulin

○, immobilized thrombomodulin; ●, immobilized albumin.

for the enhancement of protein C activation was not decreased (data not shown).

Discussion

Ebert and Kim[16] reported that it was difficult to develop the anticoagulant activity of heparin immobilized on Sepharose 6MB without a spacer arm. Heparin inhibits thrombin with antithrombin III, but does not enhance the thrombin-catalyzed protein C activation, and, hence, does not suppress the further generation of thrombin. On the other hand, it has been reported that the fibrinolytic enzyme urokinase was immobilized on several materials. The immobilized urokinase retained fibrinolytic activity.[17-19] However, urokinase activates plasminogen regardless of the activation of the coagulation cascade and the thrombus formation, and hence, there is the possibility of excessive activation of plasmin. The present study shows that a new antithrombogenic material is produced by the immobilization of thrombomodulin, which has anticoagulative characteristics different from those of heparin and urokinase.

Immobilized thrombomodulin rapidly inhibited the procoagulant activity of thrombin, and enhanced the thrombin-catalyzed protein C activation. Activated protein C has a strong anticoagulant activity. This anticoagulant activity is mainly due to the proteolytic activity of activated protein C on coagulation factors Va and VIIIa. Factors Va and VIIIa are cofactors for prothrombin and factor X activation, respectively. The anticoagulant activity of immobilized thrombomodulin–thrombin complex probably depends upon the thrombin concentration in plasma, because it is considered that the thrombin bound to immobilized thrombomodulin reversibly dissociates[7] when the thrombin concentration in plasma decreases.

Thrombomodulin is unusually stable to heat. The activity of immobilized thrombomodulin was not decreased by incubation at 37 °C for 2.5 h in citrated plasma, and the immobilized thrombomodulin prolonged the plasma recalcification time of citrated plasma (Table I). These results suggest that the activity of immobilized thrombomodulin is manifested in plasma. Repeated washing with buffer containing 0.5 M NaCl did not remove thrombomodulin from Sepharose 4B. The stability of immobilized thrombomodulin in plasma also indicates that the detachment of covalently bound thrombomodulin hardly occurs in plasma.

Our results suggest that the immobilization of thrombomodulin enables the development of an antithrombogenic material which rapidly inhibits the procoagulant activity of thrombin and supresses the further generation of thrombin through protein C activation. Since these abilities of thrombomodulin-immobilized materials are expressed only when thrombin is generated in plasma, it is expected that undesired effects would not occur. Our present results suggest that the immobilization of thrombomodulin provides a functional biomaterial which converts thrombin from a procoagulant to an anticoagulant enzyme. This work may lead to the development of an excellent antithrombogenic biomaterial.

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