Antithrombotic Effect of Recombinant Human Thrombomodulin on Thrombin-Induced Thromboembolism in Mice

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Antithrombotic effect of recombinant human thrombomodulin in mice, both in vitro and in vivo, was studied. The soluble recombinant human thrombomodulin was expressed in Chinese hamster ovary cells and purified from the conditioned medium by a modification of the conventional method. Recombinant thrombomodulin prolonged thrombin clotting time for mouse plasma in a dose-dependent manner. Thrombin was injected into the lateral tail vein of mice and caused acute thromboembolism. All mice injected with thrombin died of thromboembolism; however, preinjection with recombinant human thrombomodulin neutralized the lethal effect of thrombin in a concentration-dependent manner. Histologic examination showed that fibrin deposits were found in all large and small arteries in the lung from mice injected with thrombin; however, fibrin deposits were not detected in any large arteries from the mouse preinjected with thrombomodulin.

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MATERIALS AND METHODS

Materials. Human thrombin was purchased from Sigma (St Louis, MO). Methotrexate (MTX) was purchased from Wako Pure Chemical Industry (Osaka, Japan). Boc-Leu-Ser-Thr-Arg-MCA, a fluorogenic substrate for activated protein C, was purchased from the Protein Research Foundation (Osaka, Japan). Dihydorofolate reductase (DHFR)-deficient CHO cells were obtained from Dr L.A. Chasn (Columbia University, New York, NY). Male Balb/c mice (8 to 12 weeks old) were purchased from Japan SLC, Shizuoka.

Construction of TMD123 expression vector. The cDNA encoding domain 1, 2, and 3 of human thrombomodulin (TMD123) was constructed by site-directed deletion mutagenesis and inserted into the expression vector pSV2 as previously described. The resulting plasmid was designated pSVTMD123.

Cell culture, DNA transfection, and amplification. Plasmid pSV2TMD123 and pSV2dhfr were cotransfected into CHO DHFR cells by the calcium phosphate method. After two-steps amplification, 0.2 μg/mL MTX-resistant colony CHO-5C6G was isolated and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% (vol/vol) fetal calf serum, and the conditioned medium containing TMD123 was harvested and pooled.

Purification of recombinant protein. TMD123 was purified from the CHO-5C6G cells' conditioned medium by a modification of a previously described method using Q-Sepharose Fast Flow (Pharmacia,Upspsala,Sweden), DIP-TB (diisopropylphosphorylthrombin-agarose), and gel filtration (Pharmacia; Superose 12).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purified recombinant protein was analyzed by SDS-PAGE with 8.3% running and 4.0% stacking gel. The gel was stained with Coomassie Brillant Blue R-250 (Sigma).

Enzyme immunoassay of recombinant human thrombomodulin. The amount of recombinant human thrombomodulin was determined by the sandwich-type enzyme immunoassay using rabbit anti-human thrombomodulin immunoglobulin (IgG F(ab')2); coupled to β-D-galactosidase, prepared as described.

Protein C activation assay. The ability of thrombomodulin to accelerate thrombin-catalyzed protein C activation was measured as...
previously described. One unit of thrombomodulin activity was defined as 1 nmol of activated protein C formed/mL/min.

Coagulation assay. Blood was taken from the ventricles of mice into a plastic syringe containing 0.1 vol of 3.8% Na₂-citrate dihydrate. Platelet-poor plasma (PPP) was prepared by centrifugation at 150 x g for 15 minutes at 25°C. Thrombin clotting time (TCT) for PPP was measured using a coagulometer (KC10; Amerung, Lebrinksweg, FRG) according to manufacturer’s instructions.

Thrombin-induced thromboembolism in mice. Human thrombin (3,300 NIH U/mg) was injected over 3 seconds into the lateral tail vein of mice at several doses (0, 25, 50, and 75 U/0.05 mL/mouse), and the dose that caused embolic death within 15 minutes was determined. Recombinant thrombomodulin (TMD123) was injected into the lateral tail vein of mice at 0.05 mL/mouse at several thrombomodulin concentrations (0, 0.6, 1.2, and 2.4 mg/mL) 3 minutes before the injection of thrombin. Control mice received saline. For histopathologic examination, each lung was fixed in 10% formalin and embedded in paraffin, and sections were stained by the phosphotungstic acid-hematoxylin method.

RESULTS

Preparation of recombinant human thrombomodulin. Plasmid DNA carrying the coding sequence for domains 1, 2, and 3 (TMD123) of human thrombomodulin was transfected into CHO cells, and a high-producing cell line was established by gene amplification techniques using methotrexate. The cells were cultured in DMEM, and the conditioned medium containing recombinant thrombomodulin (1.0 µg/mL) was harvested. Purified recombinant thrombomodulin was obtained from the culture medium using ion exchange chromatography, DIP-TB chromatography, and gel filtration. SDS-PAGE of the purified fraction under reduced and nonreduced conditions showed a single major band. The molecular weight of the reduced form was estimated to be 90 kD, based on the migration pattern of molecular weight markers (Fig 1).

Effect of recombinant thrombomodulin on thrombin clotting time. To examine the effect of recombinant human thrombomodulin on mouse plasma, TCT stimulated by human thrombin for mouse plasma was determined. Recombinant human thrombomodulin prolonged TCT in a dose-dependent fashion, as shown in Fig. 2.

Effect of recombinant thrombomodulin on thrombin-induced thromboembolism in mice. Several doses of human thrombin (0, 25, 50, and 75 U/mouse) were injected into the lateral tail vein of mice. All mice injected with more than 50 U of thrombin died of acute thromboembolism, whereas those injected with 0 or 25 U of thrombin survived; thus, 50 U of thrombin was injected as a lethal dose in this experiment. Recombinant thrombomodulin was injected into mice at doses of 0, 30, 60, and 120 µg/mouse 3 minutes before thrombin injection. One hundred twenty micrograms of thrombomodulin corresponded to a 2.4-fold mol/L excess of the 50 U of thrombin injected. As shown in Table 1, all mice injected with a 2.4-fold mol/L excess of thrombomodulin over thrombin survived, and the thrombomodulin effect was dose-dependent. Those mice that survived the thrombin injection lived for over 2 weeks.

Histologic examination of mice lungs in which 50 U of thrombin was injected showed a large number of fibrin

| Table 1. Effect of TMD123 on Thrombin-Induced Thromboembolism in Mice |
|------------------------|------------------|------------------|------------------|
| TM/T                  | Survival Ratio (%) |
| 0                     | 0                | 0                |
| 0.6                   | 20               | 90               |
| 1.2                   | 80               | 80               |
| 2.4                   | 100              | 100              |
| Control               | 100              | 100              |

Recombinant thrombomodulin was injected into the mice at the molar ratio indicated. Three minutes thereafter, human thrombin (50 U/mouse) was injected into the mice. In the control experiment, a 2.4 mol/L excess of thrombomodulin over thrombin was administered and saline was injected instead of thrombin. Five mice were used at each dose.

Abbreviations: TM, thrombomodulin; T, thrombin.
Deposits in all large and small arteries stained by the phospho-tungstic acid-hematoxylin method described in the text. Conversely, fibrin deposits were not detected in large arteries in the lungs from mice preinjected with 60 μg or more of thrombomodulin, but fibrin deposits remained in some small arteries (Fig 3).

**DISCUSSION**

We demonstrated the effectiveness of the administration of recombinant thrombomodulin in preventing thrombin-induced thromboembolism in mice. The soluble recombinant human thrombomodulin (TMD123), which contains domains 1, 2, and 3, was expressed in CHO cells and purified from the conditioned medium, and migrated as a single major band in SDS-PAGE. This recombinant thrombomodulin protected mice from thrombin-induced thromboembolism. The effective dose (survival rate: 100%) of thrombomodulin under the conditions used was a 2.4-fold mol/L excess of thrombomodulin over thrombin (120 μg/mouse). Kumada and Abiko developed a synthetic thrombin inhibitor.
MD805 effectively prevented thromboembolism using similar mouse model induced human thrombin, and the effective dose (survival rate: 100%) of MD805 was 500 µg/mouse. Mice died within 10 minutes to 2 hours after thrombin injection (50 U/mouse) in their model, while all mice died within 10 minutes in our model. Thus, there are some differences of symptoms between these models, but both recombinant human thrombomodulin and MD805 are significantly effective in preventing thromboembolism using mouse model. To examine the efficacy of these drugs in more detail, they should be compared in the same model at the same time. Kumada and Abiko\(^4\) also reported that mouse thrombomodulin from mouse lung prolonged survival time of mice injected with human thrombin in a dose-dependent manner; histologic examination of mouse tissue after thrombin injection showed many fibrin deposits, but cross section of the lung from thrombomodulin-treated mice was not shown. In this study, histologic examination showed that thromboembolism was caused by fibrin deposits in lung arteries, and recombinant thrombomodulin prevented the formation of fibrin deposits in the major lung arteries.

Kurosawa et al\(^5\) and Stearns et al\(^6\) reported that an elastase-digested fragment of thrombomodulin, containing from first to sixth EGF-like structures of the second domain, and its cyanogen bromide-degraded fragment containing third to sixth EGF structures, showed protein C activated cofactor activity. We also demonstrated that the last three EGF-like structures is the minimum region necessary for protein C activating cofactor activity and anticoagulant activity.\(^7\) Therefore, we would expect that injected thrombomodulin was effective enough as an anticoagulant, even if it was digested with serum proteinase, such as elastase.

Recombinant human thrombomodulin prolonged human thrombin-stimulated clotting time in mouse plasma. This indicates that human thrombin formed fibrin clot from mouse fibrinogen and recombinant human thrombomodulin prevented the formation of the fibrin clot. Therefore, it is suggested that recombinant human thrombomodulin be bound to human thrombin to inhibit procoagulant activity in mice. These results indicate that recombinant human thrombomodulin is a potent antithrombotic agent for both in vitro and in vivo use.

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