Plasma Thrombomodulin in Health and Diseases

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Sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblot analysis of plasma thrombomodulin concentrate revealed that four degraded forms of thrombomodulin with different molecular weights are present in plasma. Plasma concentrations of thrombomodulin in patients with various diseases were measured by two methods of enzyme-linked immunosorbent assay using monoclonal antibodies. One method measures intact thrombomodulin and degraded forms of thrombomodulin; the other does not detect the two smaller degraded forms of thrombomodulin present in plasma. The results indicated that thrombomodulin was increased in the circulating blood of patients with disseminated intravascular coagulation syndrome, pulmonary thromboembolism, adult respiratory distress syndrome, chronic renal failure, or acute hepatic failure. The differences values obtained by the two methods indicate that the increase of plasma thrombomodulin found in these patients was mainly due to an increase of the smaller fragments of degraded forms, suggesting that the release of thrombomodulin from endothelial cells was accelerated in various disease states by proteolytic activity generated on the surface of the endothelium and may be removed from the circulation mostly by the kidneys and liver.

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MATERIALS AND METHODS

Collection of blood samples. Venous blood was collected into 3.2% sodium citrate (9 parts blood: 1 part anticoagulant) using a 10-mL syringe with a 21-gauge needle. Plasma was obtained by centrifuging the blood at 1,500g for 30 minutes. The plasma samples were frozen and stored at −80°C until the assay.

After informed consent was obtained, plasma samples were obtained from normal healthy donors (12 male, 10 female) and from patients with disseminated intravascular coagulation syndrome (DIC, n = 19), adult respiratory distress syndrome (ARDS, n = 9), pulmonary thromboembolism (n = 11), stable interstitial pneumonia (n = 4), lung cancer (n = 14), chronic renal failure (n = 10), compensated liver cirrhosis (n = 11), and hepatic failure (n = 8; two cases of fulminant hepatitis and six cases of advanced liver cirrhosis with hepatocellular carcinoma).

Thrombomodulin. Thrombomodulin prepared from human placenta by the previously described method was further purified by high-performance liquid chromatography. The purified thrombomodulin appeared as a single peak in a region of 100- to 150-kD proteins in gel filtration with Ultrogel AcA 34 (Pharmacia, Uppsala, Sweden) using buffer containing 0.05% Tween 20 or 0.01% Lubrol. Increase of the concentrations of the detergents used did not appreciably change the elution volume, suggesting that the thrombomodulin was monomeric in the buffer used for the assay in the present study.

Antibodies to thrombomodulin. Polyclonal antibody to human thrombomodulin was kindly supplied by Dr T. Yamaji, Bio-Medical Research Laboratories, Teijin Ltd, Iwakuni, Japan.

Three monoclonal antibodies (MoAbs)—KA-2, -3, and -4—to human thrombomodulin were used for the assay of thrombomodulin. The functional properties of these antibodies were previously described. The antibodies bind to different epitopes on the thrombomodulin molecules and do not interfere with each other's bindings. KA-3 and -4 bind to protease (elastase or trypsin)-treated thrombomodulin as well as native intact thrombomodulin, but KA-2 does not bind to protease-treated thrombomodulin. Calcium ions are required for the maximum binding of KA-4 to native thrombomodulin.

Horseradish peroxidase (HRP)-conjugated antibody. Conjugation of the antibodies with HRP (Sigma Chemical Co, St Louis) was performed by the method of Nakane and Kawai.
Antibody-coupled Sepharose. Sepharose-4B (Pharmacia, Sweden) was activated with cyanogen bromide (CNBr) by the method of Cuatrecasas, and MoAb KA-3 or polyclonal antibody to thrombomodulin was linked to theagarose to give a final IgG concentration of around 5 mg/mL of agarose.

Thrombomodulin-depleted plasma. Five milliliters of plasma was mixed and incubated with 1 mL of polyclonal antibody-coupled Sepharose for 12 hours, and thrombomodulin-depleted plasma was separated by centrifugation.

Concentration of plasma thrombomodulin by immunoaffinity chromatography. p-Aminodiphenyl methane sulfonyl fluoride-HCl (Wako Chemicals, Tokyo) was added to 15 mL of plasma to obtain a final concentration of 5 mmol/L. After incubation for 30 minutes, the plasma was dialyzed against 3 L of Tris-buffered saline (TBS) (0.02 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4). After the dialysis, the plasma sample was diluted to 100 mL with TBS and applied onto a 2-mL column of KA-3 or polyclonal antithrombomodulin antibody-conjugated Sepharose 4B (5 mg IgG/mL) equilibrated with TBS. The column was washed with 100 mL each of TBS, Tris-buffered saline (pH 7.4) 1 mol/L NaCl containing 0.05% Tween 20, and TBS, sequentially. Thrombomodulin was finally eluted from the column with 20 mL of Tris-buffered 1 mol/L NaCl containing 2 mol/L sodium thiocyanate (NaSCN). The eluted thrombomodulin was concentrated to 0.5 mL by means of Millipore CX-10 (Millipore Corp, MA) ultrafiltration or by evaporation under vacuum.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a slab gel was carried out using a separating gel of acrylamide (10%) containing 0.1% SDS and a stacking gel of 4% acrylamide containing 1% SDS according to the method of Laemmli. Molecular weight standards (rabbit muscle phosphorylase b, 97.4 Kd; bovine serum albumin, 66.2 Kd; hen egg white ovalbumin, 42.7 Kd; bovine carbonic anhydrase, 31 Kd; and soybean trypsin inhibitor, 21.5 Kd) were obtained from Bio-Rad Laboratories (Richmond, CA).

Immunoblotting technique. After separation by SDS-PAGE, the proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon Transfer, Millipore) as described by Matsuda. After the transfer, the membranes were blocked by incubating them in Tris-buffered saline containing 1% gelatin for 1 hour, washed with the washing buffer (0.05 mol/L Tris-HCl, 0.5 mol/L NaCl, 0.05% Tween 20, 5 mmol/L CaCl₂, pH 7.5), and incubated with HRP-conjugated polyclonal or MoAbs (1 μg/mL) dissolved in the washing buffer for 16 hours at 4°C. The membranes were then washed with the washing buffer and subjected to color development by soaking the membranes in a solution of 0.01% 3-aminoh-9-ethylcarbazol (Sigma), 4% dimethyl-formamide, and 0.05% H₂O₂ in 0.05 mol/L acetate buffer, pH 5.

Measurement of thrombomodulin. An enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of plasma thrombomodulin. Polyvinyl chloride microtiter plates (Nunc-Immuno Plate, Nunc, Denmark) were coated with KA-3 at 10 μg/mL in 0.05 mol/L carbonate buffer, pH 9.6, for 2 hours at 37°C. The plates were washed three times with TBS containing 0.05% Tween 20 and 5 mmol/L CaCl₂, pH 7.4 (TBS-Tween-Ca).

Standard purified thrombomodulin was diluted with TBS-Tween-Ca containing 1% bovine serum albumin (Sigma) or thrombomodulin-depleted plasma to obtain various concentrations of thrombomodulin (from 0.01 ng/mL to 5 ng/mL) and 100-μL aliquots of these solutions were added to the coated wells. Plasma was diluted various times with TBS-Tween-Ca, and a 100-μL aliquot of the diluted plasma was added to the coated wells of the same plate. The plate was incubated for 16 hours at 37°C and then washed three times with TBS-Tween-Ca.

Subsequently, 100-μL aliquots of HRP-conjugated KA-2 or KA-4 (0.1 μg/mL) was applied to each well and incubation performed for 4 hours at 37°C. After washing with TBS-Tween-Ca three times, 100-μL aliquots of the solution containing 0.01% H₂O₂ and 0.4 mg/mL orthophenylenediamine (Sigma), dissolved in 0.075 mol/L citrate-sodium phosphate buffer pH 5.0, were added to each well and incubation carried out for 30 minutes at 37°C. The reaction was stopped by adding 50 μL of 4.5 mol/L H₂SO₄ to each well, and the absorbance at 492 nm was measured by a Micro Plate Reader (Toyo Soda, Tokyo). The plasma thrombomodulin concentration was estimated according to the thrombomodulin standard curve constructed with purified thrombomodulin. When dilutions of purified thrombomodulin were made with thrombomodulin-depleted plasma for the construction of the standard curve, the absorbance obtained was dependent on the concentration of plasma protein and the absorbance was low when the plasma used was not properly diluted. When thrombomodulin-depleted plasma had been diluted more than 20 times with TBS-Tween-Ca, the standard curve obtained was identical with that constructed by dilution of purified thrombomodulin with TBS-Tween-Ca containing 1% albumin (Fig 1). Accordingly, plasma was diluted 25-fold with TBS-Tween-Ca for the assay.

Although no clot formation was observed in plasma diluted with TBS-Tween-Ca, it may be possible that activation of the blood coagulation enzyme cascade by calcium ions present in TBS-Tween-Ca takes place during the incubation, and activated coagulation factors influence the assay. This possibility was ruled out when an assay was performed using TBS-Tween-Ca containing enough heparin to suppress activation of the coagulation cascade; the addition of heparin did not alter the assay results.

Because the epitope recognized by KA-4 is located in proximity to a thrombin-binding site, there was a possibility that thrombomodulin bound to thrombin may not be measured by the present method using KA-4. To test this possibility, various amounts of thrombin were added to a fixed amount of purified thrombomodulin, and the concentration of thrombomodulin was measured by ELISA using KA-4. The values obtained were the same regardless of the amounts of thrombin added, indicating that thrombomodulin in the form of a complex with thrombin can be measured equally by the method using KA-4. The thrombin-thrombomodulin complex, if present, probably was dissociated by the dilution buffer or the washing buffer containing 0.05% Tween, and thrombomodulin thus liberated from the complex may have reacted with KA-4.

Statistical analysis. Student's t analysis for unpaired samples was used with the two-tailed test. A P value of >.05 was considered to represent a statistically nonsignificant change.

RESULTS AND DISCUSSION

Thrombomodulin was separated and concentrated from plasma by immunoadfinity chromatography using KA-3 coupled to Sepharose 4B. The concentrate was then analyzed by SDS-PAGE followed by immunoblot analysis using the monoclonal antibody KA-2 or -4. As shown in Fig 2, four protein bands were recognized by KA-4. To exclude the possibility that degradation of thrombomodulin occurred during the procedures, purified thrombomodulin was added to plasma to achieve a concentration of 1 μg/mL plasma, which is 100 times higher than the normal plasma level, and thrombomodulin was separated and analyzed by the same procedures without prior concentration. Only one band corresponding to the original purified thrombomodulin was visible by staining (Fig 2), indicating that the four bands recognized by KA-4 was not due to degradation during the procedures. Plasma thrombomodulin was also concentrated...
by immunoaffinity chromatography using polyclonal antibody to thrombomodulin, and analyzed in the same way using polyclonal or MoAbs (Fig 3). Four bands with the same mobilities as those of the bands seen in Fig 2 were recognized by KA-4 as well as polyclonal antibody, indicating that the four molecular forms represent nearly all the thrombomodulin present in plasma. All four protein bands were recognized by KA-4, whereas only two bands with larger molecular sizes were recognized by KA-2 (Figs 2 and 3). These results are in accordance with the previous findings that KA-2 recognizes intact thrombomodulin but does not recognize protease (trypsin or elastase)-degraded major fragments, whereas KA-4 recognizes protease-degraded forms as well as intact thrombomodulin.

Molecular weights of the four molecular forms estimated by nonreducing SDS-PAGE were 64, 60, 52, and 47 Kd. Since the molecular weight of intact thrombomodulin, estimated by nonreducing SDS-PAGE, was 71 Kd, all of these four molecular forms must be major fragments of partially degraded thrombomodulin. Particularly, the smallest form might represent a protease (trypsin or elastase)-degraded major fragment, because its molecular weight (47 Kd) is close to those of degraded fragments (45 and 42 Kd) obtained by elastase or trypsin digestion, respectively. Because the epitope recognized by KA-2 is likely located in the O-linked glycosylation site-rich domain or the transmembrane domain, the two smaller molecular forms may be devoid of these domains. These domains are located in the

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**Fig 2. Immunoblotting of thrombomodulin.** Purified thrombomodulin incubated with normal plasma was isolated by immunoaffinity with KA-3-conjugated sepharose and subjected to SDS-PAGE without reduction, followed by immunoblotting with HRP-conjugated antibody KA-4 (lane 1). Plasma thrombomodulin concentrate, obtained by immunoaffinity with KA-3-conjugated sepharose, was subjected to SDS-PAGE without reduction, followed by immunoblotting with HRP-conjugated antibody KA-2 (lane 2) or KA-4 (lane 3). Arrows on the right indicate the bands corresponding to the four molecular forms of thrombomodulin. On the left is a molecular weight calibration scale constructed using molecular weight standards stained with coomassie brilliant blue.
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Fig 3. Immunoblotting of plasma thrombomodulin. Plasma thrombomodulin concentrate, obtained by immunoadsorption with polyclonal antithrombomodulin antibody-conjugated sepharose, was subjected to SDS-PAGE without reduction, followed by immunoblotting with HRP-conjugated antibody KA-2 (lane 1), KA-4 (lane 2), or polyclonal antithrombomodulin antibody (lane 3). Arrows on the right indicate the bands corresponding to the four molecular forms of thrombomodulin. On the left is a molecular weight calibration scale constructed using molecular weight standards stained with coomassie brilliant blue.

A moderate but significant increase of the levels measured by the KA-2 method, which represent the levels of the larger molecular forms, was also noted in chronic renal failure. Therefore, patients with renal dysfunction were excluded from the studies on the other disease groups. The total thrombomodulin levels measured by the KA-4 method were remarkably increased in patients with DIC, pulmonary thromboembolism, ARDS, and acute hepatic failure as compared with normal values. The larger molecular forms measured by the KA-2 method were also significantly increased in DIC, ARDS, and acute hepatic failure, but not in pulmonary thromboembolism.

In contrast to ARDS and pulmonary thromboembolism, there was no increase of the total thrombomodulin measured by the KA-4 method in interstitial pneumonitis and lung cancer, suggesting that the increases observed in ARDS and pulmonary thromboembolism are unique among the pulmonary diseases and caused by the accelerated release of...
thrombomodulin from injured pulmonary capillary endothelial cells occurring in these disease states.

In patients with stable interstitial pneumonitis, lung cancer, or compensated liver cirrhosis, the levels measured by the KA-4 method were not statistically different from the level of the control. However, a significant ($P < .01$) decrease of the levels measured by the KA-2 method was noted in patients with lung cancer, and a less significant ($P < .05$) decrease was noted in patients with interstitial pneumonitis and liver cirrhosis.

Because the difference between the values obtained by the two methods represents the concentration of the two smaller molecular forms of thrombomodulin (Fig 2), the proportion of the two smaller molecular forms to the total thrombomodulin was calculated by dividing the difference by the value obtained by the KA-4 method, which measures the total thrombomodulin, and was expressed as a percentage (Fig 5).

In the control group, the two smaller molecular forms constituted an average of 43% of the total thrombomodulin in the plasma. In various disease states, however, the two smaller molecular forms constituted a significantly ($P < .01$) higher percentage of the total than in the normal state: 55% in DIC, 60% in ARDS, 66% in interstitial pneumonitis, 69% in lung cancer, 68% in chronic renal failure, and 62% in compensated liver cirrhosis. The value of 58% in pulmonary thromboembolism was less significantly ($P < .05$) higher than normal.

An increase of percentage of the smaller molecular forms in the total thrombomodulin in DIC, ARDS, pulmonary thromboembolism, and chronic renal failure indicates that the increase of the plasma thrombomodulin observed in these patients was mainly caused by the increase of the smaller molecular forms.

These findings suggest that the release of the smaller molecular forms from endothelial cells was accelerated in these disease states. In chronic renal failure, retention of thrombomodulin may be an additional factor contributing to the increase of circulating thrombomodulin if plasma thrombomodulin is cleared mainly by the kidneys. In DIC or pulmonary thromboembolism, activated proteolytic enzymes in the coagulation-fibrinolysis system may be responsible for the release of thrombomodulin. In ARDS, proteases released from leukocytes that had adhered to the endothelial cells might have split the surface thrombomodulin and released it into the circulation.

In contrast to the aforementioned disease states, the percentage of the smaller molecular forms in the total thrombomodulin in acute hepatic failure was not statistically different from that in the normal state (Fig 5), indicating that the larger molecular forms and the smaller molecular forms were increased in parallel in acute hepatic failure. It is speculated that the liver is an organ partially contributing to the clearance of plasma thrombomodulin, and a rapid and severe deterioration of liver function causes a retention of thrombomodulin in the plasma, although chronic and moderate liver dysfunction does not affect thrombomodulin levels as seen in compensated liver cirrhosis (Fig 4). In this connection it is of interest to note that the liver was the major site of clearance of intravenously injected thrombomodulin in mice.

In interstitial pneumonitis, lung cancer, and liver cirrhosis, there was no significant change of the total thrombomodulin, but the larger molecular forms measured by the KA-2 method were significantly decreased (Fig 4), resulting in an increase of the percentage of the smaller molecular forms (Fig 5). Different mechanisms of release or removal of the two classes of molecular forms may presumably be involved in changing the proportion of the two classes of molecular forms in these disease states, but the detailed mechanisms are not known.

From the results obtained in the present study, we suggest that the measurement of plasma thrombomodulin can be used to assess the state of endothelial cell injuries if renal function is normal and liver function is not severely deteriorated, but further studies are needed to support this proposal.

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ADDENDUM

After submission of this paper, a report addressing enzyme immunoassay of thrombomodulin has been published. We have confirmed the findings of Ishii et al that six molecular forms of plasma thrombomodulin were detected by SDS-PAGE under reduced conditions, whereas four molecular forms were found under nonreduced condition in the present study. They reported that the mean plasma level of thrombomodulin was 35.2 ng/mL, whereas our estimate was 11.8 ng/mL. The cause of the discrepancy is not known.

REFERENCES


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