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Disclosures

None.

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REGULAR ARTICLE

Unbalanced expression of ADAMTS13 and von Willebrand factor in mouse endotoxinemia

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Abstract

Introduction: Secondary ADAMTS13 deficiency may occur in septic patients. The expression of ADAMTS13 in mouse endotoxinemia was studied.
Methods: The blood and mRNA expression levels of ADAMTS13 and von Willebrand factor were measured in lipopolysaccharide-injected mice.
Results: The plasma ADAMTS13 activity in wild-type mice was significantly decreased at 2 h after lipopolysaccharide injection, and this decrease in ADAMTS13 activity preceded the decrease in ADAMTS13 mRNA expression in the liver and continued for 24 h. However, no decreases in the plasma ADAMTS13 activity after lipopolysaccharide injection were observed in mice pretreated with a neutrophil elastase inhibitor or in plasminogen-deficient mice, suggesting that the decrease in ADAMTS13 activity was processed efficiently by the coordinated actions of plasmin and neutrophil elastase. von Willebrand factor mRNA was abundantly expressed in the lung and moderately in the kidney, but showed relatively low expression in the liver without lipopolysaccharide injection. However, von Willebrand factor mRNA expression in the liver was significantly increased after lipopolysaccharide injection and this high expression level continued for 24 h after the injection. The von Willebrand factor and ADAMTS13 mRNA expression levels in these organs changed in the opposite manners following lipopolysaccharide administration. Furthermore, the

Abbreviations: ADAMTS 13, a disintegrin-like and metalloprotease with thrombospondin type 1 repeats; VWF, von Willebrand factor; TTP, thrombotic thrombocytopenic purpura; LPS, lipopolysaccharide; PAI-1, plasminogen activator inhibitor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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blood von Willebrand factor level increased after lipopolysaccharide administration, in contrast to the decrease in the blood ADMTS13 level after lipopolysaccharide administration.
Conclusion: These data suggest that imbalance between the blood von Willebrand factor and ADAMTS13 levels may occur in endotoxinemia, and that this may partly contribute to the thrombotic state associated with endotoxinemia.

Deficiency of a disintegrin-like and metalloprotease with thrombospondin type 1 repeats (ADAMTS) 13, a von Willebrand factor (VWF)-cleaving protease [1–5], is observed in most patients with thrombotic thrombocytopenic purpura (TTP). This deficiency is thought to be responsible for platelet aggregation and microthrombus formation in the circulation, which subsequently develop into typical thrombotic microangiopathies in humans [6–9]. In most cases of TTP, the ADAMTS13 deficiency is caused by either genetic defects in the ADAMTS13 gene (familial TTP, Upshaw–Schulman syndrome) or the presence of autoantibodies against ADAMTS13. However, decreased blood levels of ADAMTS13 have also been reported in a variety of other disease states, and thus it remains possible that secondary deficiency of ADAMTS13 may partly account for the development of microthrombus formation in other disease states besides TTP [10–14]. Indeed, we previously showed that severe secondary ADAMTS13 deficiency could take place in sepsis-induced disseminated intravascular coagulation (DIC) and was correlated with the development of renal injuries [14]. The possible mechanisms for the decreased ADAMTS13 level in septic patients may be decreased synthesis of ADAMTS13 due to liver injury and cleavage of ADAMTS13 by proteases such as plasmin, thrombin and neutrophil elastase [14]. To confirm these hypotheses, we investigated the expression of ADAMTS13 and VWF in mice treated with a lipopolysaccharide (LPS) injection. We found that the blood ADAMTS13 level decreased prior to the decrease in ADAMTS13 mRNA expression in the liver after LPS administration, while the blood VWF level and VWF mRNA expression in the liver were increased by LPS administration in the opposite manners to the changes in ADMTS13. We further provide evidence that the decrease in the blood ADAMTS13 level could be due to cleavage by the coordinated actions of neutrophil elastase and plasmin. These data suggest that imbalance between the blood VWF and ADAMTS13 levels may occur in sepsis, and that this may partly contribute to the thrombotic state in septic patients.

Materials and methods

Animal experiments

Plasminogen-deficient ($plg^{-/-}$) mice with targeted destruction of the plasminogen gene were previously reported and generously provided by Dr. P. Carmeliet (University of Leuven, Leuven, Belgium) [15]. C57BL/6 wild-type mice were purchased from SLC Inc. (Hamamatsu, Japan). Both types of mice were maintained under standard lighting conditions in a clean room. All surgical procedures were carried out in accordance with the guidelines approved by the Institutional Animal Care and Concern Committee at Jichi Medical University. Male $plg^{-/-}$ and wild-type C57BL/6 mice were used in this study. LPS (Sigma-Aldrich Japan, Tokyo, Japan) in saline was intraperitoneally injected into the wild-type mice (5–25 μg/g body weight, $n=8$) or the $plg^{-/-}$ mice (25 μg/g body weight, $n=4$) under anesthesia with isoflurane. Before and after the LPS injection, blood samples were drawn from the cervical vein plexus of the mice and mixed with a 1/10 volume of 3.8% sodium citrate, followed by platelet-poor plasma preparation by centrifugation. Mice were sacrificed under aesthesia and their organs were obtained after LPS injection. Some mice were injected with sivelestat sodium hydrate (*N*-[2-[4-(2,2-dimethylpropionyloxy)phenylsulfonylamino]benzoyl] aminoacetic acid; Ono Pharmaceutical Co., Tokyo, Japan) prior to the LPS injection to inhibit neutrophil elastase.

Determination of ADAMTS13 activity

The ADAMTS13 activities in mouse plasma were measured using a previously described method [16]. Briefly, 1–8 μL of normal human plasma or mouse plasma in 100 μL of Bis–Tris (pH 6.0) containing 25 mM $CaCl_2$ and 0.005% Tween 20 was mixed with FRETS-VWF73 (Peptide Institute, Osaka, Japan) in 100 μL of the same buffer in 96-well microtiter plates (Greiner Japan, Tokyo, Japan), and changes in the fluorescence (excitation: 320 nm; emission: 460 nm) at 37 °C were monitored using a SPECTRAMAX GEMINI (Molecular Devices Co., Sunnyvale, CA). The mouse ADAMTS13 activities were calculated from standard curves created with normal human plasma and mouse plasma without LPS injection. The mouse ADAMTS13 activities in the plasma after LPS injection were expressed as percentages relative to the activity in plasma obtained before LPS injection.

Quantification of mouse VWF

The levels of mouse VWF were quantified using the STALIA Test for VWF (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Analysis of VWF multimers in mouse plasma

The VWF multimers in mouse plasma were analyzed by SDS-agarose gel electrophoresis according to the previously described method [8,12,14].

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Detection of ADAMTS13, VWF, thrombomodulin and PAI-1 mRNAs in mouse organs

The ADAMTS13, VWF, thrombomodulin and plasminogen activator inhibitor 1 (PAI-1) mRNA expression levels were evaluated by RT-PCR. RNA was isolated from mouse organs using an RNA isolation kit (RNeasy Protect kit; Qiagen Inc., Valencia, CA). After DNAse I (Amplification Grade; Invitrogen, Carlsbad, CA) and heat treatments, the RNA samples were subjected to RT-PCR using primer pairs for ADAMTS13 (sense: 5′-GCCTCTCTTCACACACTT-3′; antisense: 5′-TATTCCACACGGTAGTCCTC-3′), VWF (sense: 5′-CTTCCTGGAAAAGTGTTGGATCTC-3′; antisense: 5′-CTGGAATGT-CTCATCTTGCTTCAG-3′), thrombomodulin (sense: 5′-AGCAGA-CTGTGACCCTAA-3′; antisense: 5′-ATTCGCCTTGACTGCACT-3′), PAI-1 (sense: 5′-CCGGAATGTGGTCTTCTCTC-3′; antisense: 5′-GCAGTTCCACGACGTCATAC-3′) and GAPDH (sense: 5′-GTCGGTGTGAACGGATTT-3′, antisense: 5′-CGTGAGTGGAGTCA-TACTGGAA-3′) and an RT-PCR kit (SuperScript One-Step RT-PCR System; Invitrogen). PCR amplification was carried out for 25 cycles.

Quantification of mouse ADAMTS13 mRNA in the LPS- injected mice (n=8) was carried out by the real time PCR using a TaqMan Universal PCR Master Mix-containing RT-PCR kit (Applied Biosystems, Foster City, CA) and a PRISM 7700 (Applied Biosystems, Foster City, CA). The amounts of mouse ADAMTS13 mRNA were standardized against the GAPDH mRNA.

Blood chemistry analyses

The blood transaminase and creatinine levels in the LPS-injected mice (n=8) were measured using commercial kits for transaminases and creatinine (Wako Pure Chemicals Industries Ltd., Tokyo, Japan), respectively.

Results

ADAMTS13 levels in mice after LPS injection

The ADAMTS13 levels in mouse blood were quantified before and after LPS injection (5–25 μg/g body weight). The blood ADAMTS13 levels in mice were not altered significantly with low and middle doses (5–15 μg/g body weight) of LPS (data not shown). When a high dose (25 μg/g body weight) of LPS was injected, the plasma ADAMTS13 activity of wild-type mice was significantly decreased at 2 h after LPS injection (Fig. 1), while the ADAMTS13 mRNA expression level in the liver was decreased at 5 h after LPS administration (Fig. 2). These data indicate that the decrease in the blood ADAMTS13 activity took place prior to the decrease in ADAMTS13 synthesis in the mouse liver at 5 h after LPS administration. In our previous study of the ADAMTS13 levels in patients with sepsis-induced DIC, we proposed that the observed decrease in the blood ADAMTS13 level could partly be caused by cleavage of ADAMTS13 by proteases such as neutrophil elastase, thrombin and plasmin [14]. To explore the possibility that the decrease in ADAMTS13 activity was caused by proteolytic cleavage by neutrophil elastase, sivelestat (20 μg/g body weight), a synthetic neutrophil elastase inhibitor used to treat acute lung injuries in septic patients, was injected intravenously into mice prior to LPS administration. Sivelestat inhibits mouse neutrophil elastase in the same manner as human neutrophil elastase, but does not inhibit plasmin, thrombin, kallikrein or trypsin [17]. Injection of sivelestat prevented the LPS-mediated decrease in ADAMTS13 activity, suggesting that neutrophil elastase partly participates in the proteolytic cleavage of ADAMTS13. Based on the results of previous *in vitro* experiments [18], plasmin and thrombin are also candidate proteases that may cleave ADAMTS13 under pathological conditions *in vivo*. Since LPS administration causes activation of the coagulation system followed by secondary fibrinolysis, $plg^{-/-}$ mice were used to study the effect of plasmin on ADAMTS13. The ADAMTS13 levels in $plg^{-/-}$ mice were not decreased after LPS injection, suggesting that plasmin may also play a role in ADAMTS13 degradation in vivo. These data raised the possibility that degradation of ADAMTS13 may be processed efficiently by the coordinated actions of plasmin and neutrophil elastase. $Plg^{-/-}$ mice were so susceptible to LPS that they died within 5 h after LPS injection, and therefore analyses of samples from $plg^{-/-}$

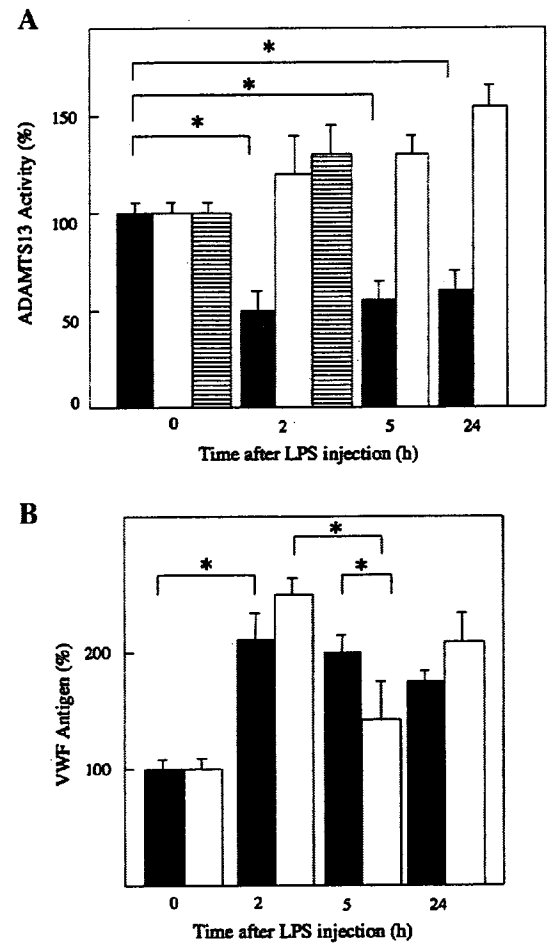

Figure 1 Blood ADAMTS13 and VWF levels in mice after LPS injection. The ADAMTS13 activity in mouse plasma obtained from LPS-injected mice was quantified as described in the Materials and methods. The mouse ADAMTS13 activities and VWF protein levels were expressed as percentages relative to the corresponding basal levels before LPS injection. (A) ADAMTS13 activities in mice after LPS injection (closed bars, n=8), sivelestat-pretreated mice after LPS injection (open bars, n=8) and $plg^{-/-}$ mice after LPS injection (hatched bars, n=4). (B) VWF levels in mice after LPS injection (closed bars) and sivelestat-pretreated mice after LPS injection (open bars). *Statistically significant difference by Student's t-test (p<0.05).

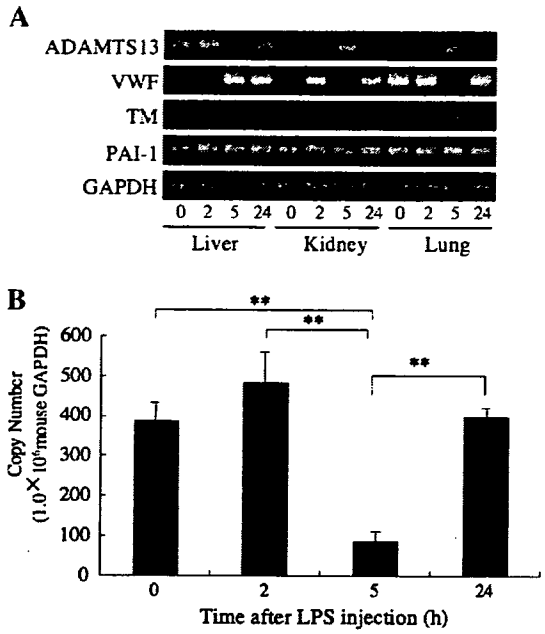

Figure 2 ADAMTS13 and VWF mRNA expression levels after LPS administration *in vivo*. (A) RT-PCR detection of ADAMTS13 and VWF mRNAs in mouse organs after LPS injection. As controls, the mRNA expressions of thrombomodulin (TM), PAI-1 and GAPDH were simultaneously analyzed by RT-PCR. The representative data were shown. (B) Quantification of ADAMTS13 mRNA expression levels in the mouse liver after LPS injection (n=8). The levels were standardized by the corresponding GAPDH mRNA level. **Statistically significant difference by Student's t-test (p<0.01).

mice at later time periods (5–24 h after LPS injection) were not possible. Since secondary fibrinolysis is dependent on fibrin formation, discrimination between the effects of thrombin and plasmin on ADAMTS13 cannot be achieved once thrombin generation is suppressed.

Expression of ADAMTS13 mRNA after LPS administration in vivo

ADAMTS13 is synthesized by liver stellate cells and the liver is thought to be the primary source of ADAMTS13 in the circulation. In addition, vascular endothelial cells in other organs may also be able to synthesize ADAMTS13 [19,20], and ADAMTS13 has been detected in platelets [21]. In accordance with these data, ADAMTS13 mRNA was detected in the liver, kidney and lung. ADAMTS13 mRNA expression in the liver was decreased to approximately one-fifth of the basal level before LPS injection at 5 h after LPS administration (Fig. 2). Analysis of alanine aminotransferase (ALT/GPT) in LPS-injected mice revealed that the blood ALT levels increased by approximately 3-fold after LPS injection (before LPS injection: 10.4±2.6 U/L; 5 h after LPS injection: 29.8±9.1 U/L), suggesting that liver injuries occurred after LPS injection but were not severe. Thus, the decrease in ADAMTS13 mRNA expression in the liver could be due to a direct effect of acute inflammation on the liver stellate cells. In contrast to these changes in ADAMTS13 mRNA expression in the liver, the ADAMTS13 mRNA expression levels in the kidney and lung increased at the same time, suggesting that ADAMTS13 gene expression in liver stellate cells and vascular endothelial cells in other organs could be differently regulated.

Changes in blood VWF levels and VWF mRNA expression after LPS administration in vivo

The thrombotic state may partly be caused by unbalanced expression of coagulation factors and their inhibitors. This may also be the case for VWF-dependent platelet adhesion. Thus, changes in the blood VWF levels and VWF mRNA expression were simultaneously studied in LPS-injected mice. The blood VWF level was increased by approximately 2-fold at 2 h after LPS injection and this high VWF level persisted for 24 h (Fig. 1). VWF mRNA was expressed abundantly in the lung and moderately in the kidney, but showed relatively low expression in the liver without LPS injection. However, VWF mRNA expression in the liver was significantly increased after LPS injection and this high level of VWF mRNA expression continued for 24 h after LPS injection (Fig. 2). VWF mRNA expression was also increased in the lung and kidney. Furthermore, the VWF and ADAMTS13 mRNA expression levels in these organs changed in the opposite manners after LPS administration (Fig. 2). These changes of VWF and ADAMTS13 mRNA expression induced by LPS were consistently observed in the mice. The mRNA expressions of GAPDH, PAI-1 and thrombomodulin were studied as internal controls. In accordance with previous reports [22,23], PAI-1 mRNA expression was increased at 2 h after LPS administration and continued for 24 h, while thrombomodulin mRNA expression was decreased at 2 h after LPS, supporting our earlier findings that changes in the VWF and ADAMTS13 mRNA expressions occurred under these experimental conditions. The data suggest that the blood VWF and ADAMTS13 levels become unbalanced after LPS administration, and that this imbalance may partly contribute to the creation of the thrombotic state in severe inflammation. When sivelestat was injected prior to LPS administration, the blood VWF level was increased by more than 2-fold at 2 h after LPS injection, similar to the case for LPS injection alone, and then decreased significantly to the level that was 1.5-fold higher than the basal level before LPS injection at 5 h after LPS injection, while the blood VWF level stayed at the 2-fold higher level relative to the basal level after LPS injection alone.

Analysis of VWF multimers in LPS-injected mice

The VWF multimer patterns were analyzed by SDS-agarose gel electrophoresis, in addition to quantification of the VWF protein levels. The intensity of each VWF multimer on the immunoblot increased after LPS administration (Fig. 3). Unusually large VWF multimers were detected in mice after LPS administration (Fig. 3). In particular, the presence of unusually large VWF multimers in LPS-injected mice was apparent in plasma obtained at 2 h after LPS injection. The amount of these unusually large VWF multimers was decreased in sivelestat-pretreated mice at 5 h after LPS injection, but did not change significantly without sivelestat-pretreatment. These findings were in accordance with the observation that the blood VWF level changed from the 2.5-fold increased level to the 1.5-fold increased level with sivelestat-pretreatment at 5 h after LPS injection. These data support the hypothesis that unbalanced expression of VWF and ADAMTS13 may cause the persistent presence of unusually large VWF multimers in the circulation, which in turn contribute to the development of the thrombotic state in severe inflammation.

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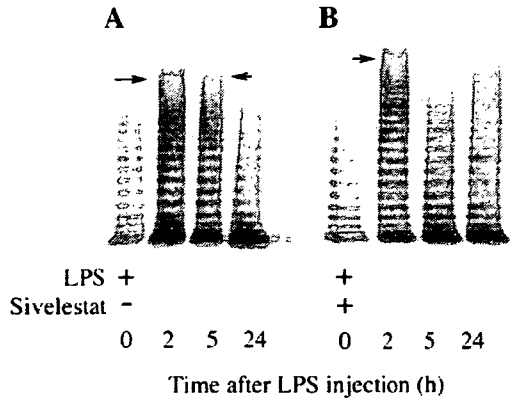

Figure 3 Analysis of VWF multimers in mouse plasma after LPS injection. The VWF multimer patterns were analyzed by SDS-agarose gel electrophoresis followed by western blotting. Typical changes in the VWF multimers in mice after LPS injection (panel A) and in sivelestat-pretreated mice after LPS injection (panel B) are shown. Arrows indicate unusually large VWF multimers found in LPS-injected mice.

Changes in blood ALT (GPT) and creatinine levels after LPS injection

The blood levels of ALT (GPT) and creatinine in mice after LPS injection with or without sivelestat-pretreatment were quantified. The creatinine levels were increased by approximately 1.6-fold at 24 h after LPS injection in mice without sivelestat-pretreatment, but this effect was not observed in sivelestat-pretreated mice (Fig. 4). Similarly, the ALT levels were increased by approximately 3-fold following LPS administration without sivelestat-pretreatment, but this effect was not observed in sivelestat-pretreated mice. These data suggest that inhibition of neutrophil elastase can prevent the development of renal and liver injuries in mouse endotoxinemia.

Discussion

ADAMTS13 has been shown to play an important role in VWF processing [1–15,24–26]. ADAMTS13 may cleave unusually large multimers of VWF on the endothelial cell surface, thereby preventing entrance of these unusually large multimers into the circulation [1–15,24–26]. Without this processing, the unusually large multimers of VWF secreted by endothelial cells would enter the circulation and initiate platelet thrombus formation, which in turn would cause the development of thrombotic microangiopathies [6]. The important physiological role of ADAMTS13-catalyzed cleavage of unusually large VWF multimers in humans is supported by reports that patients with primary ADAMTS13 deficiency caused by defects in the ADAMTS13 gene or by having autoantibodies against ADAMTS13 develop TTP [1–15,24–26].

In our previous study, we showed that severe secondary ADAMTS13 deficiency could develop in patients with sepsis-induced DIC and was correlated to the development of renal failure [14]. Here, we performed animal experiments to extend our previous study and investigate whether unbalanced expression of VWF and ADAMTS13 would occur after proteolytic inactivation of ADAMTS13 upon acute inflammation. According to a previous study, ADAMTS13 mRNA expression in vitro is not influenced by cytokines [27]. However, ADAMTS13 mRNA

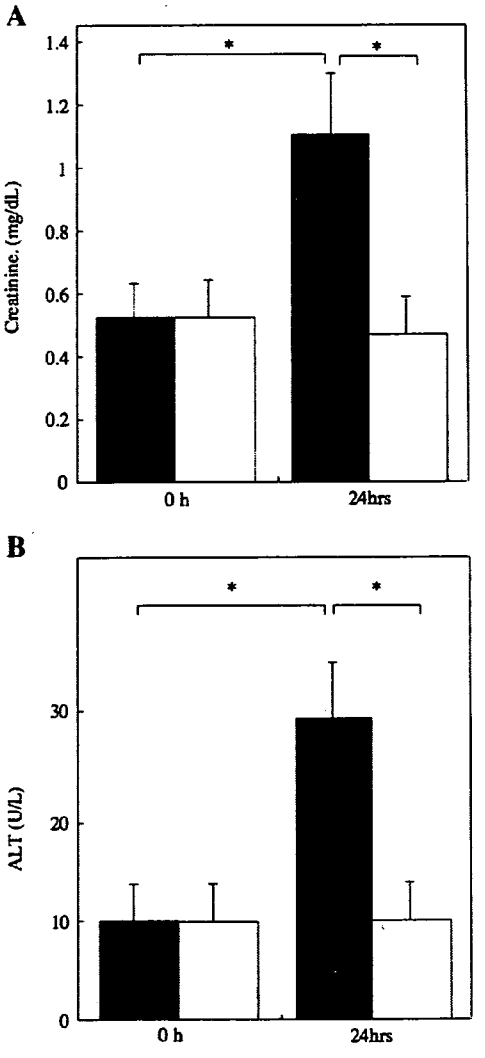

Figure 4 Changes in blood ALT (GPT) and creatinine levels after LPS injection. The blood levels of creatinine (panel A) and ALT (GPT) (panel B) in mice at 24 h after LPS injection with (open bars, $n=8$) or without (closed bars, $n=8$) sivelestat-pretreatment are shown. The values are means ± SD. *Statistically significant difference by Student's t-test ($p<0.05$).

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expression in mice in vivo was transiently decreased after LPS administration accompanied by mild liver injury, suggesting that ADAMTS13 synthesis in liver stellate cells may be altered in disease states such as sepsis. The present study revealed also that the VWF and ADAMTS13 mRNA expression levels in the liver, lung, and kidney changed in the opposite manners after LPS administration. Precise analysis of the promoter elements of the ADAMTS13 gene is the future study to elucidate the mechanisms of the ADAMTS13 and VWF mRNA expression changes. The blood ADAMTS13 activity was decreased prior to the decrease in ADAMTS13 synthesis in the liver, and this could be due to proteolytic cleavage by the coordinated actions of plasmin and neutrophil elastase, thereby supporting the hypothesis that severe secondary ADAMTS13 deficiency caused by sepsis-induced DIC may be partly due to proteolytic cleavage.

Sepsis may be the most common pathogenic disease leading to the development of DIC, and the associated endotoxemia and high cytokine levels in the circulation are thought to induce tissue factor expression, which in turn initiates fibrin microthrombus formation in the circulation. In addition, LPS and inflammatory cytokines may stimulate cells, resulting in upregulation or downregulation of a variety of genes. For example, PAI-1 gene expression is upregulated [22], while thrombomodulin gene expression is downregulated [23], by these molecules. The present study has revealed that ADAMTS13 mRNA expression is transiently decreased after LPS administration associated with mild liver injury in mice. VWF mRNA expression has been shown to increase during inflammation [28,29]. In accordance with previous studies, VWF mRNA expression levels in the kidney and lung were relatively high and increased at 2 h after LPS administration. Although VWF mRNA expression in the liver was relatively low compared with the levels in the lung and kidney, it was significantly increased after LPS administration and the increased VWF expression continued for 24 h. The blood VWF levels were increased by approximately 2-fold at 2 h after LPS administration. This initial increase in VWF could partly be due to release of VWF from vascular endothelial cells. Increased synthesis of VWF in endothelial cells, including those in the liver, may explain the sustained high blood VWF levels after LPS administration [28,29]. These data also suggest that unbalanced expression of VWF and ADAMTS13 may occur in severe infection, and that this may partly contribute to the pathological thrombus formation in sepsis. Inhibition of neutrophil elastase in mouse endotoxinemia resulted in a partial correction of the unbalanced expression of ADAMTS13 and VWF and prevented organ injuries under our experimental conditions.

The decreased blood ADMATS13 level in mice was transient and not as severe as that seen in patients with sepsis-induced DIC. These discrepancies may be due to species differences in susceptibility to LPS and the duration of the inflammation. The mechanism of the coordinated effects of plasmin and neutrophil elastase on ADAMTS13 has not yet been elucidated. It is possible that plasmin cleaves α1 proteinase inhibitor and/or that neutrophil elastase cleaves α2 plasmin inhibitor. These actions may lead to suppressed inactivation of neutrophil elastase and plasmin by their inhibitors, thereby resulting in ADAMTS13 cleavage by neutrophil elastase and plasmin.

In conclusion, there was a decrease in the ADAMTS13 level and an increase in the VWF level in the circulation after LPS administration to mice. The decrease in ADAMTS13 may be due to degradation of ADAMTS13 by the coordinated actions of neutrophil elastase and plasmin, which may partly contribute to the unbalanced expression of VWF and ADAMTS13 and the development of the thrombotic state.

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REGULAR ARTICLE

Dysfibrinogen Kagoshima with the amino acid substitution γThr-314 to Ile: Analyses of molecular abnormalities and thrombophilic nature of this abnormal molecule

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KEYWORDS
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Thrombosis

Abstract

Introduction: Emerging lines of evidence have suggested that certain dysfibrinogens present a significant risk of thrombosis.
Patient/methods: The thrombophilic nature of a new-type of dysfibrinogen Kagoshima identified in a 36-year-old female with deep vein thrombosis during the postpartum period was studied.
Results/discussion: Based on the analyses of the patient fibrinogen and the fibrinogen genes, fibrinogen Kagoshima was shown to have the amino acid substitution of γThr-314 to Ile that resulted in impaired function and hypofibrinogenemia. Polymerization of fibrin monomers derived from patient fibrinogen was severely impaired with a partial correction in the presence of calcium ions, causing very low clottability and delayed cross-linking of patient fibrin catalyzed by activated factor XIII. Because of the low clottability, a large amount of soluble fibrin was formed upon thrombin treatment, resulting in an increase of thrombin in the soluble fraction. Additionally, tPA-mediated plasmin generation on fibrin was impaired and calcium-ion-dependent integrity of the γ-chain D domain of Kagoshima fibrinogen was perturbed. The presence of many tapered-fiber ends inside the tangled fibrin

Abbreviations: tPA, tissue-type plasminogen activator; FDP, fibrin/fibrinogen degradation product; EDTA, ethylene diamine tetraacetic acid.

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networks, observed by scanning electron microscopy, suggested early termination of fibrin polymerization and the structural alteration.
Conclusion: These data suggest that fibrinogen Kagoshima is dysfunctional, giving rise to formation of fibrinolysis-resistant soluble fibrin polymers and entrance of soluble fibrin associating with thrombin to the circulation, partly accounting for the thrombophilic nature of the affected fibrinogen and fibrin molecules.

Fibrinogen is a 340-kDa plasma protein that plays a variety of roles in blood coagulation, platelet aggregation, cellular and matrix interactions, inflammation, wound healing, and cancer [1,2]. Fibrinogen is composed of two identical molecular halves, each being composed of an Aα-, a Bβ-, and a γ-chains, encoded by three independent genes. During synthesis in the liver, these chains are translated, processed, and assembled to the mature molecules [3]. Mutations in the fibrinogen genes can result in molecular and functional defects, such as defects in fibrinopeptide release, polymerization defects, cross-linking of fibrin molecules, and fibrinolysis of fibrinogen/fibrin molecules (dysfibrinogenemia), or decreased levels of fibrinogen in blood (afibrinogenemia and hypofibrinogenemia), depending on the abnormality [4–6]. Even a single amino acid substitution of fibrinogen can cause hypofibrinogenemia, indicating that not only intracellular transport and secretion but also assembly of fibrinogen molecules can be affected by a single amino acid substitution. It is also possible that an amino acid substitution could cause hypercatabolism of the abnormal molecules [6,7].

Although functional sites in the fibrinogen/fibrin molecules and their interactions were precisely determined [see reviews [1,2]], analyses of molecular and functional abnormalities of dysfibrinogens would greatly facilitate elucidation of the structure–function relationships of fibrinogen/fibrin, fibrin polymerization mechanisms, and pathophysiology of abnormal thrombus formation [4]. Depending on the molecular abnormality, patients with dysfibrinogen may show bleeding tendencies, thromboembolisms, or no apparent symptoms. Most patients with dysfibrinogenemia have no apparent symptoms. Many cases with fibrin polymerization defects caused by an amino acid substitution in the D domain of the γ-chain have been reported, but only a few of these individuals have a bleeding tendency [8]. Patients with certain types of dysfibrinogen [4,5,8–13] have thrombosis. Mechanisms for the thrombophilia of dysfibrinogen may be assigned to defective binding of thrombin to abnormal fibrin due to the molecular defect in the low-affinity thrombin-binding site, causing increased thrombin levels, or the defective stimulatory function of abnormal fibrin in tissue-type plasminogen activator (tPA)-mediated fibrinolysis caused by mutations in the plasminogen binding sites and/or tPA binding sites [4,5,8–13]. Here we report a new dysfibrinogen with the amino acid substitution γThr-314 to Ile, resulting in liberation of soluble fibrin associating with thrombin from clots and formation of fibrin clots with altered structure caused by conformational defects in the vicinity of the D:D association site, the high-affinity calcium-binding site, and the tPA-binding site of the γ-chain.

Materials and methods

Analysis of purified fibrinogen

This study was approved by the Bioethics Committees of Jichi Medical University and Kagoshima University. All the samples were obtained with informed consent according to the Declaration of Helsinki. Fibrinogen was purified from the plasma of normal subjects or patient's plasma as described previously [14]. Clotting of patient-derived fibrinogen was compared with that of normal fibrinogen by thrombin time in the absence or presence of calcium. The aggregation profiles of normal and patient-derived fibrin monomers were monitored at 350 nm according to the method of Gralnick as described previously [12,14]. Release of fibrinopeptides A and B and the cross-linking profiles of fibrinogen γ-chains (γ-dimer formation) and α-chains (α-polymer formation) were studied as described previously [12,14].

Determination of the nucleotide sequences of fibrinogen genes

Patient genomic DNA was isolated from peripheral leukocytes by the previously described standard procedure [15] with informed consent. All the exons and exon–intron boundaries of fibrinogen genes were amplified by PCR using Bucabest DNA polymerase (Takara Co., Kyoto, Japan) and appropriate primers (Invitrogen Japan, Tokyo, Japan) [15]. PCR-amplified DNA fragments were isolated and their nucleotide sequences were determined.

Determination of thrombin in the soluble fraction of fibrin clots

Thrombin (50 nM) in 75 μL of Tris-buffered saline containing 2.5 mM calcium was mixed with increasing concentrations of Kagoshima fibrinogen (0–10 μM) in 75 μL of the same buffer containing FXIII (2 U/mL) at 37 °C for 30 min. The fibrin clots were squeezed with bamboo sticks and the supernatants were harvested. The amounts of thrombin remaining in the supernatants of the fibrin-clot samples were measured by quantifying hydrolysis of the synthetic substrate S-2238 (absorbance change at 405 nm/min). Aliquots (5 μL) of the supernatants of fibrin clots

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were taken after the incubation with Kagoshima fibrin solution [thrombin (50 nM), fibrinogen (10 μM)] and added to 200 μL of normal fibrinogen (2.94 μM), and fibrin gel formation and release of fibrinopeptides of the normal fibrinogen were analyzed after incubation at 37 °C. Aliquots of the supernatants of normal fibrin clots were added to the normal fibrinogen solution under the same conditions as the control.

Fibrin degradation by tPA-catalyzed plasmin digestion and tPA-mediated plasmin generation

TPA-mediated plasmin generation in the presence of preformed clots [16] and forming clots [12] were determined as previously described.

Plasmic digestion of fibrinogen

Plasmic digestion of fibrinogen in the presence of calcium ions was analyzed by SDS-PAGE to study the protective effects of calcium ions on the γ-chain D domain. Fibrinogen (1.47 μM) was incubated with plasmin (0.27 μM) in 20 μL of Tris-buffered saline pH 7.4 at 37 °C for 60 min in the presence of EDTA (2 mM) or increasing concentrations of calcium, and plasmic digests of fibrinogen were analyzed by SDS-PAGE.

Scanning electron microscopy of fibrin clots

To investigate the structure of Kagoshima fibrin, clots were formed on carbon-formvar-coated gold grids and processed for scanning electron microscopy using a JEOL JSM6300F Field Emission Scanning Electron Microscope (Japan Electron Optics Laboratory, Tokyo, Japan) as described previously [17].

Results

Description of the patient

A 36-year-old woman who suffered deep vein thrombosis on day 17 after delivery was admitted to a hospital for treatment. Echography revealed the presence of thrombi from the left popliteal vein to the left iliac vein, and perfusion scintigraphy showed filling defects in the left lung field, suggesting the presence of pulmonary thromboembolism. She was suspected of having dysfibrinogen based upon the coagulation tests conducted on admission, in which there was a marked discrepancy between fibrinogen levels in plasma determined by thrombin time (<0.88 μM) and the immunological method (2.16 μM) (normal range: 5.9–11.8 μM). Since the plasma fibrinogen level determined by the immunological method was decreased to approximately 1/3 of the normal level, the patient was thought to have a hypo-dysfibrinogenemia. The fibrin/fibrinogen degradation products (FDP) level, determined using polyclonal antibodies against fibrinogen, was significantly increased to 224.8 μg/mL, but the platelet counts (204×10^9/L) were normal and the D-dimer level (5.8 μg/mL), determined using a monoclonal antibody that binds specifically to the D-dimer of cross-linked fibrin but not to fibrinogen, was increased slightly, suggesting that consumption of fibrinogen was unlikely. The increase of the D-dimer level could be due to the presence of deep vein thrombosis. The significant difference between the FDP level and the D-dimer level suggested that unclottable fibrinogen/fibrin was present in patient's blood. Other coagulation parameters including the plasma levels of protein C, anti-thrombin III, plasminogen, and protein S were within normal ranges. No anti-β2-Glycoprotein I antibody was detected. The coagulation test for lupus anticoagulant was also negative, indicating that the presence of anti-phospholipid antibody syndrome was not likely. The patient's mother had similar abnormalities in her coagulation tests (decreased fibrinogen levels and increased levels of FDP), although she had no apparent history of thrombosis, suggesting that the patient was thought to have congenital dysfibrinogen that we designated as fibrinogen Kagoshima.

Abnormalities of purified fibrinogen Kagoshima and genetic abnormality of patient fibrinogen genes

The clotting time of patient-derived fibrinogen (Kagoshima fibrinogen) with thrombin was significantly prolonged to 21.7 s in the presence of calcium (control: 6.7 s) and was not measurable in the absence of calcium (control: 14.9 s). These data confirmed that fibrinogen Kagoshima was a dysfibrinogen. The apparent mobility of the Aα chain and Bβ chain polypeptides of purified fibrinogen Kagoshima on SDS-PAGE were indistinguishable from those of normal fibrinogen Aα chain and Bβ chain polypeptides (Fig. 1). There was a polypeptide band that migrated faster than the normal γ-chain on SDS-PAGE in the Kagoshima fibrinogen preparation. Western blotting analysis of purified fibrinogen preparations showed that the polypeptide band (γ^* in Fig. 1) was recognized by the γ-chain-specific monoclonal antibody JIF-25 [12], indicating the presence of a small amount of γ-chain with faster mobility on SDS-PAGE in Kagoshima fibrinogen (Fig. 1). The amount of this polypeptide band (γ^*), quantified using a densitometer, was approximately 1/4 of the normal γ-chain band of the patient's fibrinogen preparation. We performed genetic analysis of the three patient fibrinogen genes. As shown in Fig. 1, both C and T were found at position 5846 in the directly sequenced PCR-amplified DNA fragments of exon 8 derived from the patient fibrinogen γ-chain

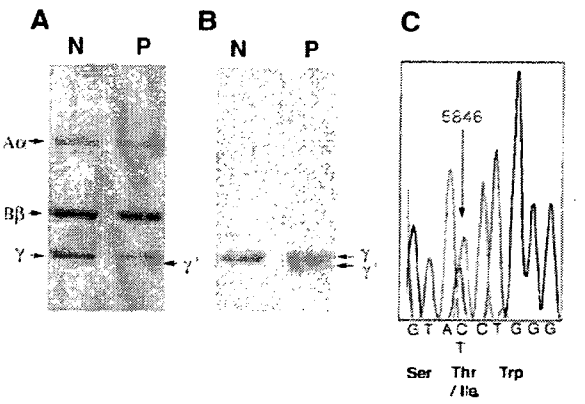

Figure 1 SDS-PAGE analysis of purified fibrinogen and nucleotide sequences of the γ-chain gene exon 8. The SDS-PAGE analysis (SDS-PAGE) of purified fibrinogen (A) and identification of the γ-chain by Western blotting (B) using the γ-chain-specific monoclonal antibody JIF-25 are shown. (C) The DNA fragments amplified by PCR for the γ-chain gene exon 8 derived from the patient were subjected to direct nucleotide sequencing. The nucleotide position at 5846 of the γ-chain gene, where both T and C were detected, is indicated by the arrow. When the DNA fragments were sequenced with the anti-sense primer G82, both A and G were detected at the same nucleotide position (not shown).

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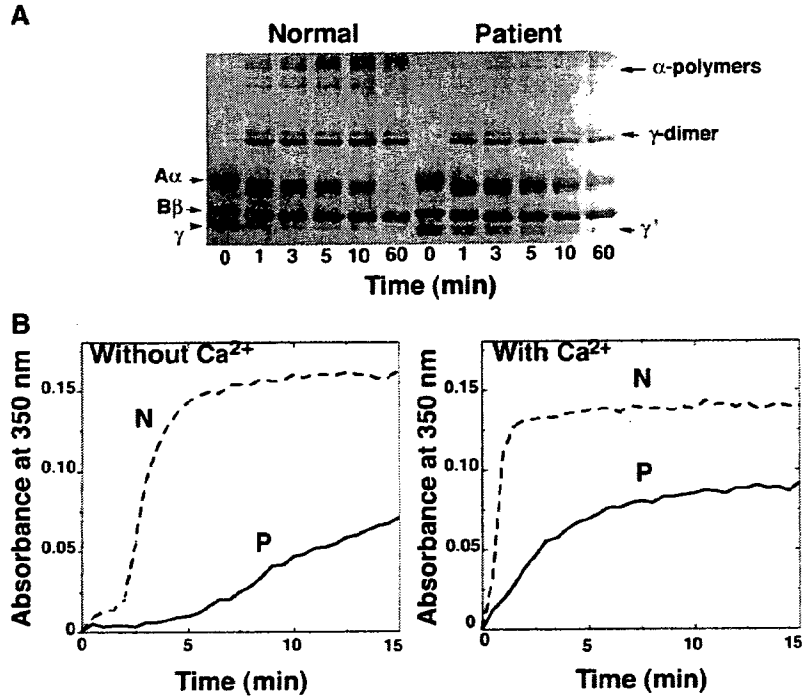

Figure 2 Analyses of fibrinopeptide release, cross-linking of fibrin, and polymerization profiles of fibrin monomers. (A) Release of fibrinopeptides A and B and formation of γ-dimer (γ-dimer) and α-polymer (α-polymer) upon thrombin treatment in the presence of factor XIII and 2.5 mM calcium was analyzed by SDS-PAGE. (B) Aggregation of fibrin derived from normal fibrinogen (dotted line) or from patient fibrinogen (solid line) at neutral pH in the absence (without Ca^{2+}) or presence (with Ca^{2+}) of 2.5 mM calcium was monitored at 350 nm.

gene, indicating that the patient had a heterozygous genetic mutation and that this mutation would result in the amino acid substitution γ Thr-314 to Ile in the affected γ-chain. In comparison with normal fibrinogen and fibrin, conversion of the Kagoshima fibrinogen Aα chain to the α-chain by thrombin was similar to that of normal fibrinogen, indicating that the release of fibrinopeptide A was not impaired, as expected (Fig. 2). Release of fibrinopeptide B from Kagoshima fibrinogen was slightly delayed compared with normal fibrinogen. This might be due to delayed polymerization. In contrast, γ-dimer formation and the α-polymer formation of the Kagoshima fibrin was significantly delayed (Fig. 2), suggesting that the amino acid substitution γ Thr-314 to Ile affected the D:D contact. It is also possible that this might be in part a consequence of the delayed polymerization. Abnormal fibrinogens with the amino acid substitution in the C-terminal γ-chain that have impaired

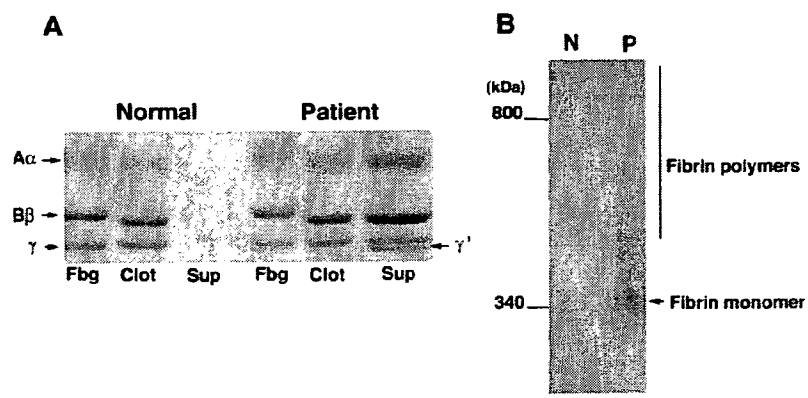

Figure 3 SDS-PAGE analysis of soluble Kagoshima fibrin. Untreated fibrinogen (Fbg), insoluble clots (clot), and soluble fractions (Sup) were analyzed by SDS-PAGE using 7.5% polyacrylamide gels under reducing conditions (A). For analysis of soluble Kagoshima fibrin formed in the presence of calcium ions, the supernatant of the normal preparation (N) and the patient fibrin preparation (P) were separated on 3% SDS-PAGE gels under non-reducing conditions (B).

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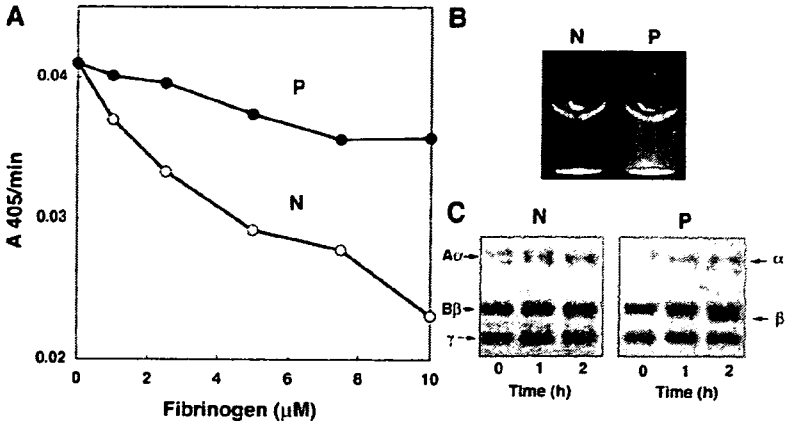

Figure 4 Determination of thrombin in the supernatant of fibrin clots. A: Thrombin was incubated with increasing concentrations of fibrinogen (N: normal fibrinogen, P: patient fibrinogen) in Tris-buffered saline containing 2.5 mM calcium and FXIII at 37 °C for 30 min. After separation of fibrin clots, the amounts of thrombin in the supernatants of clots were measured by quantifying hydrolysis of the synthetic substrate S-2238. Panel B shows gel formation of normal fibrinogen solution upon incubation with the supernatants of fibrin clots derived from normal (N) and patient fibrinogen (P). Panel C shows the SDS-PAGE analysis for conversion of normal fibrinogen to fibrin upon incubation with the supernatants of fibrin clots derived from normal (N) and patient fibrinogen (P).

fibrin polymerization but do not have delayed FXIIIa-catalyzed γ-chain cross-linking have been reported previously [8]; thus, delayed γ-chain cross-linking would mainly be caused by the amino acid substitution γThr-314 to Ile. Polymerization profiles of fibrin monomers derived from Kagoshima fibrinogen were significantly impaired (Fig. 2). In particular, polymerization of Kagoshima fibrin was severely perturbed in the absence of calcium, confirming that the patient-derived fibrinogen was dysfunctional. Even in the presence of 2.5 mM calcium ions, absorbance of the Kagoshima fibrin solution increased much more slowly than that of the normal fibrin solution, and the maximum absorbance of the Kagoshima fibrin solution was approximately 2/3 that of the normal fibrin solution, suggesting that the fibrin polymerization was severely perturbed (Fig. 2).

Analysis of soluble Kagoshima fibrin

Kagoshima fibrinogen could be converted to fibrin by thrombin treatment, but its polymerization was perturbed and there was an increase of FDP level in the patient's blood, suggesting that Kagoshima fibrin might not form solid clots and might be present as soluble fibrin. To demonstrate that soluble fibrin monomers and polymers were formed upon coagulation, normal fibrinogen and Kagoshima fibrinogen were treated with thrombin and FXIII in the presence or absence of calcium and the formed clots were squeezed to separate clots from the soluble components. Then the fibrin clots and the soluble components were analyzed by SDS-PAGE. The supernatant of the normal fibrin preparation did not contain soluble fibrin even in the absence of calcium ions (Fig. 3A). In contrast, a large amount of Kagoshima fibrin remained in the soluble fraction (Fig. 3A). Soluble Kagoshima fibrin monomers and polymers were also present in the supernatants of clots formed in the presence of calcium (Fig. 3B). The analysis of the soluble fraction containing soluble fibrin by SDS-PAGE showed that the soluble fraction of the patient's clots contained an increased amount of the abnormal γ-chain (Fig. 3A), suggesting that the polymerization reaction may be terminated once the abnormal fibrin molecules binds to the normal fibrin molecules.

Determination of thrombin in the soluble fraction of fibrin clots

Since a large amount of soluble fibrin remained in the supernatant of the Kagoshima fibrinogen derived clots (Fig. 3), thrombin might have remained in the supernatant of fibrin clots. Thus, the amounts of thrombin remaining in the soluble fractions (supernatants) of fibrin clots were quantified using S-2238. As shown in Fig. 4, the amounts of thrombin decreased in the supernatant of fibrin clots in a fibrinogen dose-dependent manner. Compared with normal fibrin clots, thrombin remaining in the supernatant of the Kagoshima fibrin clots was significantly increased. Effects of the soluble fibrin remaining in the supernatants on normal fibrinogen were studied to see if thrombin in the soluble fibrin fraction causes new fibrin formation. The two simultaneously performed experiments shown in Fig. 4B, C indicate that gel formation occurred after a 60-min incubation when the supernatant of Kagoshima fibrin clots was added to a normal fibrinogen solution, but there was no apparent gel formation when the supernatant from normal fibrin clots was added to a normal fibrinogen solution. The analysis of fibrinopeptide release from normal fibrinogen incubated with the supernatants of fibrin clots confirmed that fibrinopeptide release occurred more rapidly in the fibrinogen incubated with the Kagoshima fibrin-clot supernatant than that with the normal fibrin-clot supernatant. These data suggested that soluble fibrin associating with thrombin

Table 1 tPA-mediated plasmin generation on preformed fibrin clot

	Normal	Kagoshima	
Fibrinogen	1.63±0.27 (n=5)	1.59±0.15 (n=5)	NS
Fibrin	3.26±0.36 (n=5)	2.33±0.23 (n=5)	*p<0.05

Values are V_{max} (10^{-5}/s).
NS: not significant, *statistically significant (Student t-test).

formed upon clotting in the patient can be released into the circulation. Thrombin associated with soluble fibrin may subsequently be dissociated from soluble fibrin because of the low affinity, resulting in new fibrin-clot formation and thrombosis.

Analysis of tPA-catalyzed plasmin digestion of fibrin and tPA-mediated plasmin generation on fibrin surfaces

Since the amino acid substitution of Kagoshima fibrinogen resided in the putative tPA binding site of the D domain γ-chain, we speculated that binding of tPA to Kagoshima fibrin might be affected. Thus, tPA-catalyzed plasmin digestion of Kagoshima fibrin and tPA-mediated plasmin generation on Kagoshima fibrin surfaces were studied. Comparison of plasmic digestion of cross-linked Kagoshima fibrin with that of normal fibrin was difficult because polymerization of fibrin molecules and cross-linking of the γ-chain and α-chain were delayed (data not shown). Thus we analyzed tPA-mediated plasmin generation on preformed fibrin and forming fibrin. Plasminogen activation by tPA on Kagoshima fibrin was perturbed compared with that on normal fibrin surfaces (Table 1, Fig. 5), suggesting that Kagoshima fibrin would be resistant to physiological fibrinolysis.

Plasmic digestion of fibrinogen in the presence of calcium ions

Since the amino acid substitution of fibrinogen Kagoshima might affect the high-affinity calcium-binding site of the γ-chain D domain, calcium-ion-dependent integrity of the γ-chain D domain was thought to be altered. Kagoshima fibrinogen was studied for its resistance to plasmin in the presence of calcium ions (Fig. 6). Normal fibrinogen was converted to the fragment D1 upon incubation with plasmin and degraded further in the presence of EDTA, but no further degradation of D1 was observed in the presence of 0.05 mM calcium (Fig. 6). When Kagoshima fibrinogen was incubated with plasmin, small amounts of fragments D2A and D3 were identified in addition to fragment D1, even at the calcium concentration of 0.8 mM. These D2A and D3 fragments could be derived from the abnormal fibrinogen molecules. These data suggested that the calcium-ion-dependent integrity of the Kagoshima fibrinogen γ-chain was not sufficient to protect fragment D1 from plasmic digestion.

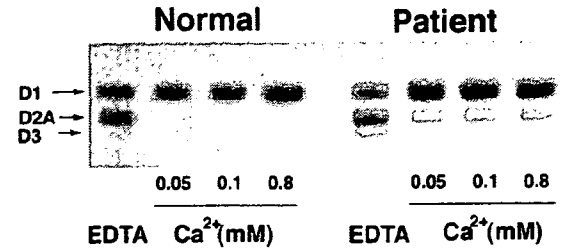

Figure 6 Plasmic digestion of fibrinogen. Normal or Kagoshima fibrinogen was incubated with plasmin in the presence of EDTA or increasing concentrations of calcium ions, and the fibrinogen fragments were analyzed by SDS-PAGE.

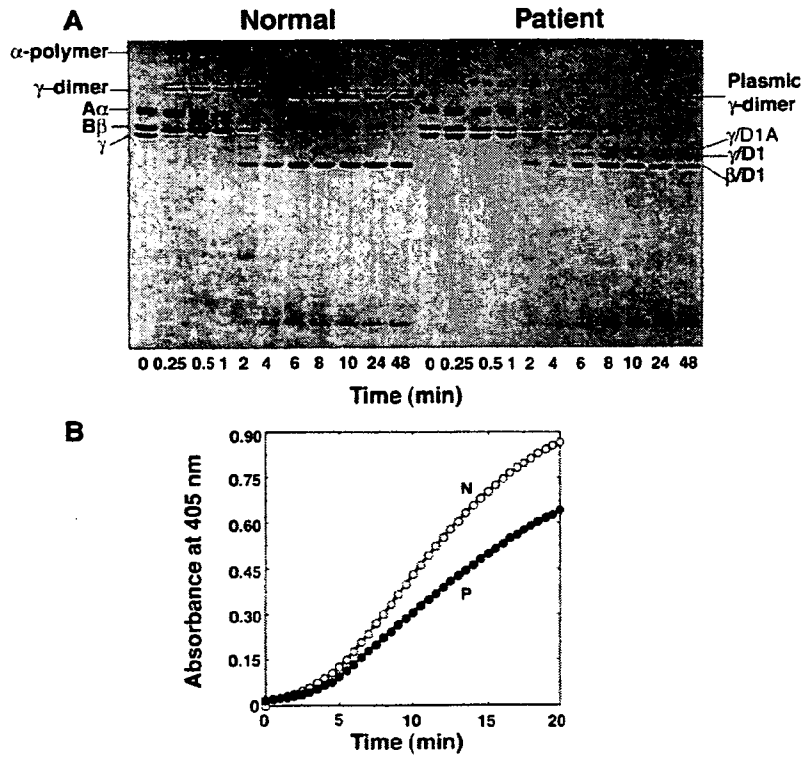

Figure 5 Analysis of tPA-mediated plasmin generation on fibrin surfaces. Normal (open circles) or patient fibrinogen (closed circles) was incubated with plasminogen, tPA, thrombin, and S-2251 in the presence of 2 mM calcium. The changes in absorbance at 405 nm were monitored. Background absorbance changes determined with samples incubated in the absence of fibrinogen at 405 nm were subtracted from the experimental values.

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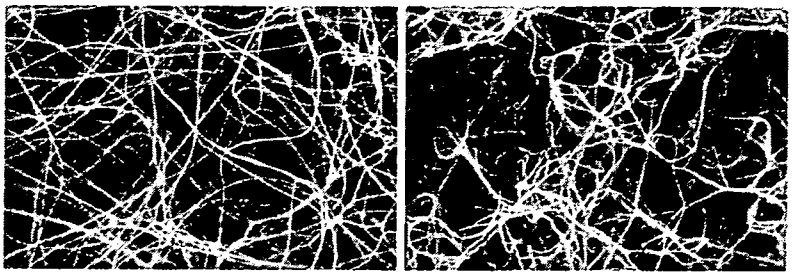

Figure 7 Scanning electron microscopy of fibrin clots. Scanning electron microscopy images of normal fibrin molecules as in your original text: "these abnormal molecules".

Scanning electron microscopy of fibrin clots

Scanning electron microscopy was carried out to investigate the structure of the Kagoshima fibrin clots. The Kagoshima fibrin clots were composed of tangled fibers with a variety of diameters and there existed many tapered-fiber ends inside the fibrin networks (Fig. 7). The presence of fiber ends in the networks might represent early termination of fibril polymerization.

Discussion

Fibrinogen Kagoshima has an amino acid substitution in which the γThr-314 is changed to Ile, and this dysfibrinogenemia was found in a 36-year-old woman with deep vein thrombosis in the postpartum period. Although the correlation of this dysfibrinogen with thrombophilia was not fully supported by the family history, analysis of purified Kagoshima fibrinogen suggested that this abnormal fibrinogen molecule has a thrombophilic nature.

Emerging lines of evidence have suggested that certain types of dysfibrinogen present a significant risk factor of thrombosis [4,5,8–13]. Mechanisms for thrombophilia of dysfibrinogen may be assigned to defective binding of thrombin to abnormal fibrin that leads to increased free thrombin levels caused by abnormalities in the low-affinity thrombin-binding site or to resistance to fibrinolysis [4,5,8–13]. The thrombophilic nature of fibrinogen Kagoshima may be accounted for by both formation of fibrinolysis-resistant fibrin and generation of a large amount of soluble fibrin associating with thrombin that results in new fibrin-clot formation. Thrombophilic dysfibrinogen has been thought to be a rare molecular abnormality because approximately 10% of dysfibrinogenemia patients are associating with thrombosis [8]. However, the incidence of thrombosis in patients with dysfibrinogen whose amino acid mutation resides in the C-terminal region of the γ-chain increases to approximately 30% [8]. We reported recently thrombophilic dysfibrinogen Tokyo V, which has the amino acid mutation of γAla-327 to Thr with an extra-glycosylation at Asn-325 [12]. Both Kagoshima fibrin and Tokyo V fibrin are resistant to tPA-mediated fibrinolysis and have a severe fibrin polymerization defect that results in formation of large amounts of soluble fibrin. Similar to fibrinogen Kagoshima, the amount of thrombin in the soluble fibrin fraction derived from Tokyo V fibrinogen is increased (data not shown). In addition to the resistance to tPA-mediated fibrinolysis of these abnormal fibrin molecules, the other shared feature of formation of large amounts of soluble fibrin containing thrombin, that may subsequently be dissociated from soluble fibrin, may also be responsible for the thrombophilic nature of dysfibrinogen with structure alterations in the tPA binding sites, the D:D interface, "a" pocket for D:E contact, and the calcium ion binding site.

The C-terminal region of the γ-chain comprises part of the D domain and has a variety of functional sites including the "a" polymerization pocket for D:E contact, the association site for D:D contact, the tPA-binding site (γ 311–336), and the high-affinity calcium-binding site (γ 318–324) [1,2]. The amino acid substitution of Ile for γThr-314 of Kagoshima fibrinogen affected these important fibrinogen/fibrin functions directly or indirectly as shown in this study. Based on the crystal structure of the D domain of fibrinogen [18], γThr-314 resides near the D:D interface and γArg-275. Thus, the substitution of Thr at this position to the hydrophobic amino acid Ile would likely strongly interfere with D:D contact. The delayed cross-linking reaction between γ-chains would support this notion. The data shown in Fig. 6 suggested that the amino acid substitution of Ile for γThr-314 of Kagoshima fibrinogen would also affect the high-affinity calcium-binding site (Fig. 6), thereby influencing indirectly D:E contact (Fig. 6). The tPA binding site in the D domain γ-chain may be altered, causing a decrease of tPA binding to Kagoshima fibrin as shown in this study. As evidenced by the scanning electron microscopy analysis showing the tangled fibers with many fiber ends in the Kagoshima fibrin

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networks, the structure of Kagoshima fibrin clots may be perturbed compared with the structure of normal fibrin clots. However, such Kagoshima fibrin clots also undergo impaired fibrinolysis due to decreased tPA-catalyzed plasmin generation, thereby partly accounting for the thrombophilic nature of this molecule.

In conclusion, fibrinogen Kagoshima, found in a 36-year-old woman with deep vein thrombosis in the postpartum period, is a hypo-dysfibrinogen with the amino acid substitution γThr-314 to Ile. The thrombophilic nature of fibrinogen Kagoshima may be accounted for by formation of fragile fibrinolysis-resistant clots and a large amount of thrombin-containing soluble fibrin caused by conformation defects in the vicinity of the D:D association site, the calcium ion binding site, and the tPA binding site of the γ-chain.

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■ 原　著　　日血外会誌 17：7–12，2008

学会セッションのテーマ変遷から見た医療水準
—静脈血栓症における医療訴訟の検討—

川﨑　富夫

要　旨：【はじめに】学会は会員にさまざまなメッセージを発信する．だが重要なのは，会員が何を受け取りどのように了解したのかである．会員が了解したメッセージは，総会活動の中に痕跡として残されている．このメッセージをどのようにとらえて利用できるのかを検討した．【方法】1999年から2006年まで心臓血管外科専門医制度を構成する3学会を対象に，静脈血栓塞栓症に関わる主要セッションのテーマ(セッション・テーマ)解析を行った．さらに，この結果を公的鑑定に使用可能かどうかを検討した．事件は1999年当時の肺塞栓症の予防として深部静脈血栓症の予防を行う注意義務があったかどうかが問われた控訴審であった．【結果】日本胸部外科学会では関連テーマがなく，日本心臓血管外科学会では2005年に治療が一度取り上げられていた．一方，日本血管外科学会では診断と治療が2001年以降5セッションで取り上げられ，深部静脈血栓症予防による肺塞栓症の予防は2006年に初めて取り上げられた．最高度の医療水準を示す学会セッションでも，深部静脈血栓症の予防による肺塞栓症の予防が取り上げられたのは2006年が初めてであったことから，それ以前の肺塞栓症の予防とは深部静脈血栓症の早期診断と早期治療であったことを明らかにできた．鑑定において1999年当時の医療水準をこのような方法で示すことができ控訴棄却となった．【結論】セッション・テーマの解析により，学会の専門性と「当時の医療水準」の「上限」を示すことができた．(日血外会誌　17：7–12，2008)

索引用語：メッセージ，セッション，テーマ，ガイドライン，訴訟

はじめに

医事関係訴訟において，被告病院の診療内容が「当時の医療水準」に達していたかどうかの観点から，司法が判断をくだす場合が多い．裁判官は医学の専門家ではないから，原告や被告双方の医師の意見書あるいは中立的立場の医師による公的鑑定書を重視する．この意見書や鑑定書は，その時代の医療水準に則った公平で正しい評価で記されるべきである．だが医療は日々発展を続けており，反省と更新の過程で，常に揺れ動くものである．そのような中では，絶対的基準など見あたらない．そのため鑑定医ごとに判断が異なり，医療水準の水準点が移動する．鑑定自体にブレが生じ，医療不信に繋がる．

医療水準の措定は会員のコンセンサスに基づくべきである．学会が主催する大会において，折々に取り上げられる主題(テーマ)とは，この医療水準を考える上で恰好の材料となる．各セッションに与えられたテーマ(セッション・テーマ)に注目すれば，テーマの繰り返しや変遷は，そのテーマの検討価値を示す指標となる．ここで議論され合意されたテーマにおける結論は，その時の医療水準を反映する．当然ながらこれは出席の各会員が了承した結論でもある．異議や意見を

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もつ会員は，総会でそれを主張する機会が認められているからである．ただしこれは医学会におけるオピニオンリーダーたちの，最高度の医療水準であることを承知しておかなければならない．一般医の医療水準は，また別の所に存在する．

今回，この学会テーマを取り上げ，そのテーマの変遷について触れることにした．医療水準の解析を行うためには，テーマの変遷を知ることが不可欠だからである．テーマがどのように出現し，どのように取り上げられ，どのように問題提起され，どのように学会員たちに受け入れられていったのか．そのテーマについて，どのように医療水準は確定していったのか，それが解明できるからである．

筆者はこの手法を公的鑑定に応用してみた．その結果，当時の医療水準を明解に示すことができ，司法の信頼を得ることができた．テーマの変遷と併せ，公的鑑定の意義，医療水準の基準についても，若干の意見を述べることとする．

対象と方法

1．セッション・テーマの選択

深部静脈血栓症と肺塞栓症に直接関係するセッションを調査検討した．また，学会のコンセンサスが形成されるセッションとして，学会総会におけるシンポジウム，ワークショップ，コンセンサスミーティング，プレナリセッション，会長要望演題，ディベートセッション，そしてパネルディスカッションを対象とした．一方，会長講演，一般演題，招請講演，特別講演，教育講演，ランチョンセミナー，モーニングセッション，イブニングセッション，インターナショナルセッションを対象から除外した．これらは見識を異とする医師同士の討論の結果の行方を一般聴衆が見届けることができず，学会のコンセンサスが形成されているといえないからである．

2．対象とした学会

血管内科が育っていない日本では，静脈疾患のほとんどを血管外科医が担当している．その多くの医師は日本血管外科学会に所属しており，専門医資格は心臓血管外科専門医のみである．この専門医制度を運営する心臓血管外科専門医認定機構を構成する3学会，つまり日本血管外科学会，日本心臓血管外科学会，日本胸部外科学会を調査対象とした．調査期間は1999年から2006年までとした．

3．鑑定事例の概略と鑑定方法

手術当時の肺塞栓症予防対策が適切であったかどうかが争われた控訴審例である．患者は1999年，ある地方都市の基幹病院にある手術目的で入院した．深部静脈血栓症のリスクは肥満と長時間手術であったが，下肢腫脹はなく深部静脈血栓症を疑われていなかった．患者は手術後に突然肺塞栓症を発症した．原告側医師の意見書には，1999年当時のガイドライン通りの予防対策を病院が怠ったことが原因であると記載されていた．そこで裁判所は，被告病院における予防対策の周知と実践の程度を，当時の当該病院規模の病院(地方の基幹病院)と比較した上での判断を，公的鑑定に求めてきた．

被告病院と同規模の病院での「当時の医療水準」を示せる客観的データは少ない．そこで，「医療水準」を形成する当時の「肺塞栓症の予防概念」を検討した．肺塞栓症の予防戦略には，「深部静脈血栓症の早期診断と早期治療」による方法と，「深部静脈血栓症そのものを予防」する方法とがある．前者は当時日本の静脈血栓症専門家が確立を急いでいた方法である．後者は欧米で行われていた方法であるが，ヘパリンの至適投与量が日本では欧米と異なることが指摘されていた．現在から遡って1999年の「当時の医療水準」が両者のいずれであったのかを，客観的データであるセッション・テーマの解析結果に基づいて検討し，その判断を司法に返すことにした．

4．ガイドラインの評価

1999年当時のガイドラインには，「深部静脈血栓症の予防をもって肺塞栓症を予防する」方法が記載されていた．このガイドラインが「医療水準」にあたるかどうかを判断するために，「ガイドライン」の定義に合致するかどうかを検討した．「EBMを用いた診療ガイドライン作成・活用ガイド」はガイドライン作成のためのガイドラインであり，医療側の立場からまとめられたものである[1]．そこで，静脈血栓症に対して，わが国で初めて学会規模でまとめられた2004年日本循環器学会の「肺血栓塞栓症および深部静脈血栓症の診断・治療・予防に関するガイドライン」が，この定義に合致しているかどうかを検討した．2004年にまとめられた代表的ガイドラインが「医療水準」に達していなければ，1999年の同内容のガイドラインも「医療水準」に達していないといえるからである．

結　果

1．セッション・テーマ

学会総会抄録集のセッション名に「深部静脈血栓症」または「肺塞栓症」が含まれるものを調査した．すると日本胸部外科学会には該当するセッションがなく，日本心臓血管外科学会は2005年に1セッション「深部静脈血栓症・肺塞栓症の治療指針」があった．一方，日本血管外科学会では2001年から計5セッションがあり，このなかで肺塞栓症の予防，診断，および治療の全てが扱われていた(**Table 1**)．

また，3学会のなかで，肺塞栓症を予防する目的で「深部静脈血栓症の予防」が初めて学会のセッション・テーマとなったのは，2006年日本血管外科学会「静脈血栓塞栓症予防ガイドラインの検証と課題」のみであった．それ以前は，「深部静脈血栓症の早期診断と早期治療」方法の確立が日本血管外科学会を中心に精力的に行われており，これにより肺塞栓症の予防がはかられていたことが明らかとなった(**Table 1**)．

2．ガイドライン

「EBMを用いた診療ガイドライン作成・活用ガイド」によれば，ガイドラインの成熟過程は第1段階が臨床専門医のみで作成したもの，第2段階は証拠に基づいて医療を高めるため臨床研究方法論の専門家が作成グループに参加したもの，第3段階は患者・消費者・関連企業などのより広い利害関係者が参加したものとに分けられる[1]．2004年日本循環器学会「肺血栓塞栓症および深部静脈血栓症の診断・治療・予防に関するガイドライン」は，この第1段階のものに過ぎない．一部の専門家のみが作成したもので，日本におけるエビデンスに乏しく，司法に対して作成責任と説明責任をとれる責任者がいない．利害相反(conflict of interest)に関する記載がなく，各学会の明確なコンセンサスも得ていない．

また，このガイドラインで引用された文献は全314編である．日本語論文50編，日本人が書いた英文論文22編，日本人以外の論文242編(全体の77%)である[2]．このガイドラインは，海外のガイドラインやデータに依存しており，欧米のガイドラインの翻訳が主体をなすと考えられる．

3．鑑定の結論

セッション・テーマを解析した結果から，1999年当時の「医療水準」において，肺塞栓症の予防とは「深部静脈血栓症の早期診断と早期治療」であったことがわかる．「肺塞栓症予防としての深部静脈血栓症予防」が学会で検討されたのは2006年以降である．また，静脈血栓症予防に関して初めて学会が作成した2004年の循環器病学会のガイドラインでさえ，欧米のガイドラインの翻訳に過ぎず，日本人の疾病構造から導き出されたものではない[2]．会員間でコンセンサスが得られたものではなく，あくまで参考であり情報提供の範囲内である．したがって，2004年よりさらに前の1999年当時に，ガイドラインとよばれていた書物のなかで「肺塞栓症予防として深部静脈血栓症予防」が触れられていても，それがわが国の当時の「医療水準」を示しているわけではない．

つまり1999年当時の肺塞栓症予防手段は，「深部静脈血栓症の早期診断と早期治療」であり，「深部静脈血栓症の予防をもって肺塞栓症の予防とする」ものではなかった．したがって，当時深部静脈血栓症の予防が行われなかったからといって，「肺塞栓症を発症させた過失が被告病院にある」とはいえないとの結論となる．この鑑定に基づき控訴棄却の判決となった．

考　察

1．学会の専門性の特徴

主要なセッションで活発に検討が繰り返される学会とそうでない学会とでは，継承される知的遺産の量が決定的に異なる．静脈疾患の専門家は，心臓血管外科専門医認定機構を構成する3学会のなかでは日本血管外科学会を中心に活動している．だがそのような専門家の多くは，さまざまな制約により日本心臓血管外科専門医の資格をとることができない．これは，静脈疾患について社会が期待する専門家は，心臓血管外科専門医とは別であることを示している．

2．医療水準

「医療水準」とは，医療のレベルの平均ではなく，また病院が守るべき最低ラインでもない．むしろ会員全体のレベルを引き上げるため，その暫定「目標」ないし司法に使用される意図としては「上限」や「最高度の医療水準」となるものである．

ところが司法は，「医療水準」の定義を「(当該)診療契約に基づき(当該)医療機関に要求される医療水準」とした．医療機関が整えるべき体制について，「そのような

Table 1 Session themes of society meetings related to venous thromboembolism

Japanese Society for Vascular Surgery

	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	Etiology, treatment, and long-term result of deep vein thrombosis	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	Deep vein thrombosis: Thorough discussion of diagnosis and treatment	–	–
Workshop	–	–	–	–	–	–	–	–
Consensus Meetng	–	–	–	–	–	–	–	–
President Demand	–	–	–	–	Treatment of pulmonary thromboembolism 1	–	–	Inspection and problems of the guideline for venous thromboembolism
President Demand	–	–	–	–	Treatment of pulmonary thromboembolism 2	–	–	–

Japanese Society for Cardiovascular Surgery

	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	–	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	–	–	–
Workshop	–	–	–	–	–	–	–	–
Forum	–	–	–	–	–	–	–	–
President Demand	–	–	–	–	–	–	Guidelines for treatment of venous thromboembolism	–

Japanese Association for Thoracic Surgery

	1999	2000	2001	2002	2003	2004	2005	2006
Symposium	–	–	–	–	–	–	–	–
Pannel Discussion	–	–	–	–	–	–	–	–
Workshop	–	–	–	–	–	–	–	–
Forum	–	–	–	–	–	–	–	–