Interaction between Human Tissue Thromboplastin and Human Antithrombin III

Keizo HIRAHARA,*[,a] Tetsuro MATSUISHI,[a] Nobuo SUZUKI[a] and Munetsugu KURATA[b]

Pharma Research Laboratories, Hoechst Japan Limited,[a] 1-3-2 Minamidai, Kawagoe, Saitama 350, Japan and Faculty of Pharmaceutical Sciences, Josai University,[b] Keyakidai, Sakado, Saitama 350-2, Japan. *Received April 21, 1989*

It is known that antithrombin III (ATIII) activity is inhibited by tissue thromboplastin (TP) in the presence of heparin. In our study on the mechanism of the inhibition, however, TP was found to inhibit ATIII activity even in the absence of heparin, indicating an interaction between ATIII and TP. We then studied effect of ATIII on the interaction between factor VII (FVII) and TP using FVII-depleted human plasma. When the mixture of FVII + ATIII + TP was incubated at 37 °C and mixed with FVII-depleted plasma, the interaction between FVII and TP was inhibited. Possible complex formation was examined by electrophoretic techniques.

In the immunoelectrophoresis, ATIII shifted toward the cathode in the presence of TP and the substance which had changed the mobility of ATIII showed TP activity after zone electrophoresis. The results indicated that TP had shifted toward the anode due to ATIII while ATIII had shifted toward the cathode due to TP. In the immunoblotting analysis, ATIII was separated into several bands in the presence of TP. ATIII antigenicity was altered in the presence of both heparin and TP but not in the presence of TP alone.

Our results strongly suggest that TP modulates ATIII activity in the initiation of the TP-mediated coagulation cascade and that in progressing coagulation ATIII participates in the inhibition of the coagulation cascade by blocking not only thrombin activity but also the interaction between TP and FVII.

Keywords antithrombin III; tissue thromboplastin; factor VII; heparin; coagulation cascade; disseminated intravascular coagulation

Antithrombin III (ATIII) is one of the most important of the physiological protease inhibitors which control and modulate the blood coagulation cascade. Its *in vivo* effect is considered to be mainly the inhibition of thrombin activity.[1,2] Heparin is known remarkably to enhance the inhibition rate of ATIII against coagulating enzymes *in vitro*.[3] ATIII is unable to inhibit completely the activity of coagulation factor Xa which has formed complexes with phospholipids and Ca^{2+}.[4] Furthermore, ATIII activity is inhibited by fatty acids, tissue thromboplastin (TP) in the presence of heparin, *etc.*[5-7] We have also confirmed in our previous study that the heparin cofactor activity of ATIII is inhibited by TP,[8] and in the course of the study we have obtained a preliminary result that the progressive thrombin inhibition activity of ATIII was depressed by the presence of TP.

On the other hand, ATIII has also been reported to combine with endothelial cells *via* heparin-like substance (Stern *et al.*)[9] and non heparin-like substance (Bartha *et al.*).[10] Both Stern's and Bartha's groups have pointed out that the dissociation of ATIII from endothelial cells occurs within a few minutes. However, these phenomena were observed only with intact cells and, as Nemerson indicated,[11] the participation of the endothelium in the initiation of coagulation seems less under normal physiological conditions; it is presumed that exposure of the endothelium to interleukin and/or tumor necrosis factor at inflammation sites and/or bacterial infection-induced lipopolysaccharide (endotoxin) or the combination of all three produces TP on the surface of endothelial cells.[12-14] In this way TP binds to FVII/FVIIa. This interaction increases the rate of activation of FX by FVII/FVIIa, initiating blood clotting by the extrinsic pathway.[15,16] ATIII does not affect FVIIa activity in the presence or absence of heparin under physiological conditions.[17,18] We have reported in the preceding study that the TP-mediated coagulation cascade in plasma was hardly affected by the presence of heparin and/or ATIII.[8] Ofosu *et al.* have reported that TP partitions ATIII–heparin complexes because of formation of complexes with heparin.[19] Based on the facts that (1) ATIII combines with fatty acids,[5,6] glycolipids,[20] steroids,[21] *etc.*, (2) TP has lipoprotein character and (3) the progressive activity of ATIII is inhibited by TP, we first undertook an examination of the interaction between human TP and human ATIII.

Materials and Methods

Materials Bovine thrombin, human fibrinogen, human placental TP (Thromborel-S®), human placental phospholipid (Pathromtin®), highly purified human ATIII, FVII-depleted human plasma, agarose and anti-human ATIII serum (rabbit) were from Behringwerke AG (FRG). Glycine, tris(hydroxymethyl)aminomethane, veronal and its sodium salt were purchased from Wako Pure Chemicals Co., Ltd. (Japan), human FVII from Sigma Chemical Company (U.S.A.), heparin from Green Cross Ltd. (Japan), electrophoresis substances for immunoblotting from Bio Rad Laboratories (U.S.A.), and polyacrylamide gel for electrophoresis from Daiichi Chemicals Co., Ltd. (Japan). Solutions of fibrinogen, heparin, ATIII, thrombin and TP were prepared with veronal buffer solution (25 mM, pH 7.6) containing 0.2% bovine serum albumin. The purity of human ATIII was calculated to be above 99% from the sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoretic pattern. The TP used here did not contain thrombomodulin detectable by our protein C assay method.[8]

Measurement of ATIII Activity We modified the method of Hensen and Loeliger.[22] A 25-μl portion each of ATIII (18 μM) and heparin (1 U/ml), 50 μl of thrombin (1.5 U/ml) and 50 μl of 50-fold dilution of TP were mixed and incubated at 37 °C for 1 min. Immediately after the incubation, 50 μl of $CaCl_2$ (25 mM) and 50 μl of human fibrinogen (10 mg/ml) were added to the reaction mixture and the clotting time was measured with a fibrin timer (Behringwerke AG).

Influence of ATIII on the Interaction between FVII and TP We modified the method of Nemerson and Clyne for the measurement of FVII activity.[23] A 50-μl portion of human FVII (0.4 μg/ml), 50 μl of TP usually used for prothrombin time measurement and 50 μl of ATIII (3 or 18 μM) were mixed and incubated at 37 °C for 1 min. After the addition of 50 μl each of FVII-depleted human plasma and $CaCl_2$ (25 mM), the clotting time was measured with a fibrin timer. Then, a mixture of 50 μl each of ATIII (3 or 18 μM) and TP was incubated at 37 °C for 1 min. To the mixture, 50 μl each of FVII (0.4 μg/ml) and FVII-depleted plasma and then 50 μl of $CaCl_2$ (25 mM) were added to measure the clotting time. The clotting times of the preincubated mixture of FVII and TP and that of FVII and ATIII were also measured in a similar manner, to serve as the controls.

Analysis by Immunoblotting Mixtures of equal volumes of ATIII (90 μM) and TP ($\times 4$, $\times 8$, $\times 16$ dilution) were subjected to polyacrylamide gel electrophoresis (PAGE) and then immunoblotting. The procedure of PAGE followed the manual of Daiichi Chemical Co. Immunoblotting was done according to the method of Renart *et al.*[24] except that Horize Blot® (ATTO Co., Japan) was used as the blotting apparatus and that the electrophoretic run was done at a fixed voltage of 11 V for 2 h.

Immunoelectrophoresis We followed the method of Grabar and William.[25] A 5 μl sample of each of the following was placed in wells of a 1% agarose plate: 1, ATIII (30 μM); 2, ATIII (90 μM) + phospholipid + veronal buffer; 3, ATIII (90 μM) + TP + veronal buffer; 4, ATIII (90 μM) + heparin (100 U/ml) + veronal buffer; 5, ATIII (90 μM) + TP + heparin (100 U/ml); 6, ATIII (90 μM) + phospholipid + heparin (100 U/ml); 7, ATIII (30 μM). After an electrophoretic run at 3 mA/cm for 90 min, rabbit anti-human ATIII serum was added to each groove and allowed to react at room temperature for 18 h to examine the precipitation lines.

Detection of TP Activity in the Extracts from Zone Electrophoresis Gel After the zone electrophoresis of a mixture of ATIII and TP, the gel was cut in 0.5-cm slices and each slice was immersed in saline solution to extract proteins. The TP activity of the extract was determined. The electrophoretic conditions were same as those stated under "immunoelectrophoresis," and 15-μl samples of the equi-volume mixture of ATIII (90 μM) and TP were put in the wells. The TP activity was measured by the method of Hirahara *et al.*[8]; 50 μl of plasma was added to mixtures of 50 μl of $CaCl_2$ (25 mM) and 50 μl of the extract from each of the 0.5-cm slices, then the clotting time was measured.

Ouchterlony's Method Samples used in the immunoelectrophoresis were tested according to the method of Nakamura and Sugiura.[26] The antigen well was 3 mm in diameter and each sample was applied to a well in a volume of exactly 6 μl with a microsyringe. The wells were allowed to stand at room temperature and observed at 4 and 12 h.

Results

Inhibition of ATIII Activity by TP The inhibition of ATIII activity by TP was examined in the presence or absence of heparin. ATIII activity was strongly suppressed by TP in the presence of heparin. In the absence of heparin, suppression was also seen (Table I).

This result raised the possibility that TP might affect ATIII directly, so the effect of ATIII on the interaction between FVII and TP was examined with FVII-depleted human plasma. As shown in Table II, incubation of ATIII (18 μM) and TP depressed the interaction of TP with FVII, which resulted in prolongation of clotting times. The prolongation was significantly different between the incubation of FVII and ATIII (18 μM) before the addition of FVII-depleted plasma with TP and incubation of ATIII (18 μM) with TP (t-test, $p<0.05$). With 3 μM ATIII, however, no inhibitory effect was seen. ATIII by itself at 18 μM prolonged the clotting time.

These results suggested the formation of a complex of TP with ATIII, especially since ATIII possesses a binding site for glycolipids, and steroid hormones and fatty acids are known to combine with ATIII.[5,6,20,21] We employed electrophoretic techniques to confirm the interaction between TP and ATIII.

Analysis of Interaction between ATIII and TP by Electrophoresis The ATIII–TP mixture was analyzed by immunoelectrophoresis. As Fig. 1 shows, ATIII shifted toward the cathode in the presence of TP and absence of heparin and, in the presence of heparin, separated into 2 fractions with high or low mobility. On addition of TP to this system, the ATIII fraction with low mobility shifted toward the cathode but the one with high mobility was not affected by TP.

Next, zone electrophoresis was done to identify the material which had changed the mobility of ATIII with TP. After an electrophoretic run, we cut the gel in 0.5-cm slices, extracted the substances from each slice, and measured the activity. As Fig. 2 shows, fraction No. 4 showed a significant difference in TP activity from the control (TP alone), which indicated the TP had shifted toward the anode due to ATIII while ATIII had shifted toward the cathode due to TP. The coagulation activity of each fraction was also confirmed to be due to TP because the clotting time of the sample using FVII-depleted plasma was equal to that of the control.

Whether ATIII and TP had formed complexes or not was examined by PAGE and immunoblotting. As Fig. 3 shows, three bands (→) appeared in the presence of TP. These results suggested that the inhibition of ATIII activity by TP might be caused by the depressed reactivity of ATIII with thrombin due to the binding of ATIII to TP.

Since ATIII changes to a so-called "open mouth" in the presence of heparin because of a structural change,[27] there is a possibility that ATIII in the presence of heparin might

Table I. Effect of Tissue Thromboplastin on Antithrombin III Activity

Sample	n	Clotting time (s)	
		Mean	S.D.
ATIII + heparin + TP	5	69.9	14.9
ATIII + heparin	5	>240	—
ATIII + TP	4	103.1	10.2
ATIII	4	136.6	17.1

A test sample was added to thrombin solution and fibrinogen solution was added to the mixture to measure the clotting time.

Table II. Effect of Antithrombin III on the Interaction between Factor VII and Tissue Thromboplastin

Sample	n	Clotting time (s)			
		ATIII (18 μM)		ATIII (3 μM)	
		Mean	S.D.	Mean	S.D.
ATIII + FVII + TP → D-plasma	4	43.3	6.1	14.6	1.1
FVII + TP + medium → D-plasma	4	14.1	1.1	—	—
ATIII + TP → FVII + D-plasma	3	43.0	5.6	14.5	0.2
FVII + ATIII → TP + D-plasma	3	35.0	2.3	14.1	1.1

D-Plasma, FVII-depleted human plasma.

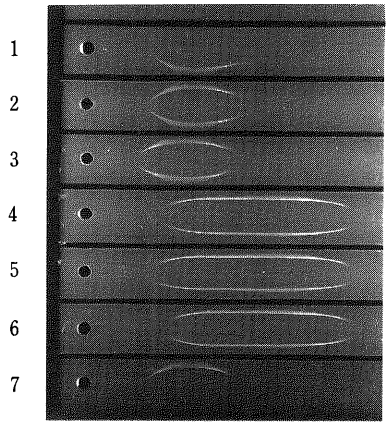

Fig. 1. Immunoelectrophoresis

1, ATIII; 2, ATIII + phospholipid; 3, ATIII + tissue thromboplastin; 4, ATIII + heparin + phospholipid; 5, ATIII + heparin + tissue thromboplastin; 6, ATIII + heparin; 7, ATIII. Anti human ATIII serum was added to each groove.

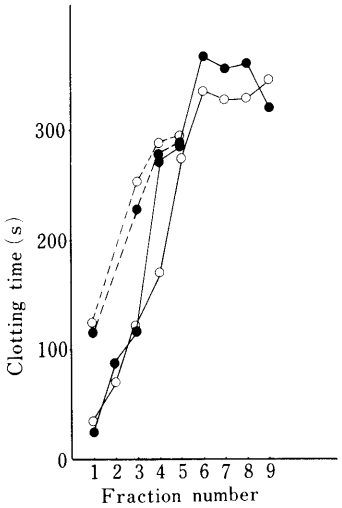

Fig. 2. Detection of Tissue Thromboplastin Activity in the Extracts from Zone Electrophoresis Gel

●, tissue thromboplastin; ○, tissue thromboplastin+ATIII. The dotted line denotes the absence of FVII in plasma.

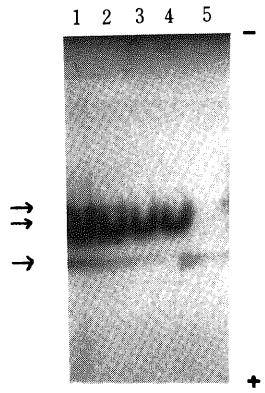

Fig. 3. Immunoblotting Analysis of Tissue Thromboplastin-ATIII

1, ATIII+tissue thromboplastin (×4); 2, +tissue thromboplastin (×8); 3, +tissue thromboplastin (×16); 4, ATIII alone; 5, tissue thromboplastin alone (×4).

bind to TP in a different manner from the binding in the absence of heparin. Therefore, we examined the antigenicity of ATIII in the presence of TP with heparin.

Reactivity of ATIII with Anti-human ATIII Antibody in the Presence of TP The reactivity of the ATIII+TP+heparin mixture with anti-human ATIII serum was compared with those of ATIII alone and the ATIII+TP and ATIII+heparin mixtures. As shown in Fig. 4, the reactivity of ATIII changed only in the presence of both TP and heparin and was hardly different from that of ATIII alone in the presence of either of the two separately. Loss in reactivity in the presence of TP and heparin was slightly restored by further incubation up to 12 h.

Discussion

Ofosu *et al.*[19] have reported that tissue factor (tissue thromboplastin) has the greatest anti-heparin activity, partitions heparin from ATIII and prevents full expression of the ATIII-dependent anticoagulant activity of heparin. In our previous experiments, on the other hand, the TP-mediated coagulation could be controlled not only by ATIII alone with heparin at physiological concentrations

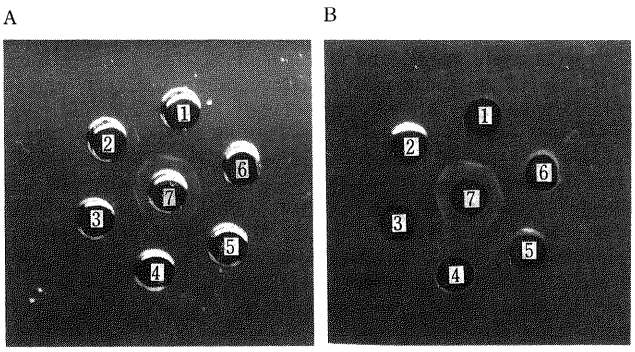

Fig. 4. Reactivity of ATIII in the Presence of Heparin and Tissue Thromboplastin against Anti-Human ATIII Antibody

1, ATIII; 2, ATIII+heparin; 3, ATIII; 4, ATIII+tissue thromboplastin+heparin; 5, ATIII; 6, ATIII+tissue thromboplastin; 7, antihuman ATIII serum. A, observation after 4-h incubation; B, observation after 12-h incubation.

but in the presence of activated protein C, and the ATIII-heparin cofactor activity was inhibited by TP.[8] Furthermore, the progressive antithrombin activity was also inhibited by TP (Table I). Heparin forms complexes with phospholipids,[28] lipoproteins,[29] and Vitamin K-dependent clotting factors.[30] Reportedly, complex formation with these substances affects the interaction between ATIII and heparin. Furthermore, ATIII itself binds to fatty acids, steroids and glycolipids.[5,6,20,21] These facts raise the possibility that the heparin cofactor activity may be inhibited by neutralization of heparin activity by TP and by the interaction between ATIII and TP. If ATIII combines with TP, the interaction between TP and FVII may be inhibited. As shown in Table II, 18 μM ATIII suppressed the interaction between FVII and TP and prolonged the clotting time. With 3 μM ATIII, however, no inhibitory effect was seen; these results suggest that the interaction between FVII and TP is not inhibited until more than a definite amount of ATIII combines with TP. According to Heimburger, in coagulation, ATIII migrates to the coagulation site and is localized there at far higher concentrations than the normal level in blood.[31] This might indicate that in the TP-mediated coagulation TP binds ATIII to facilitate coagulation at the injured site of vascular walls and plays a role so that ATIII may not inhibit thrombin activity immediately after thrombin formation even in the presence of heparin or heparin-like substances. On the other hand, the localized ATIII at high concentrations may suppress the interaction between TP and FVII (Table II). We therefore analyzed the interaction by use of electrophoretic techniques. In the immunoelectrophoresis, ATIII shifted toward the cathode in the presence of TP: from the TP activity of the extract from the gel obtained by zone electrophoresis, TP appeared to shift toward the anode in the presence of ATIII, and ATIII, toward the cathode in the presence of TP. In the immunoblotting we detected several bands, which corresponds with Rao's report that anti-TP monoclonal antibody yields three bands against human brain TP.[32] The electrophoretic data obtained in the present study (Figs. 1—3) suggest that the inhibition of ATIII activity by TP might be caused by the depressed reactivity of ATIII with thrombin due to the binding of ATIII to TP. The binding of TP to ATIII observed here is considered to be an interaction between the phospholipid moiety of TP and the hydrophobic moiety of

ATIII based on the fact that fatty acids, steroid hormones, *etc.* combine with ATIII.[5,6,21] It has already been established that TP is usually absent in circulating blood but is expressed on the membrane of many cells including the endothelium.[12-14] Several papers report the binding of ATIII to endothelial cells: a heparin-like substance and others have been proposed as binding sites.[9,10] In addition to those binding sites, TP may also be a binding site for ATIII on the surface of perturbed cells.[13]

As Fig. 4 shows, the reactivity of ATIII with anti-human ATIII antibody was lowered in the presence of TP with heparin, suggesting that TP may control the effect of ATIII in the initiation of TP-mediated coagulation in relation to the presence of heparin or heparin-like substances, since thrombin inhibition by ATIII and heparin normally occurs instantaneously. The alteration of antigenicity may support the view that TP suppresses ATIII activity in the presence of heparin.

Since many patients with leukemia, however, have been reported to show disseminated intravascular coagulation (DIC) with increases in the levels of TP from leukemic cells in blood,[33,34] ATIII activity in the plasma of those patients may be decreased as follows: (1) inhibition of progressive activity and/or heparin cofactor activity by TP; and (2) rapid consumption of ATIII for the excess coagulation. Since the decrease of ATIII activity in plasma causes DIC, treatment of DIC patients with ATIII has been the most effective medication.[35]

Though the results presented here need to be confirmed by further experiments using perturbed endothelial cells, the finding that the effect of ATIII or ATIII + heparin is controlled by TP so as to facilitate the coagulation cascade initiated by FVII and TP and then suppress the excess coagulation presents a useful area for the investigation of the fine mechanisms of blood coagulation in the human body.

Acknowledgement We wish to express our thanks to Mr. Hazelrigg of Saitama University for advice on the English manuscript.

References

1) E. W. Davie and D. J. Hanahan, "The Plasma Proteins," Vol III, ed. by F. W. Putnam, Academic Press, New York, 1977, p. 421.
2) P. C. Harpel, "Hemostasis and Thrombosis," ed. by R. W. Colman, J. Hirsh, V. J. Marder and E. W. Salzman, J. B. Lippincott Company, Philadelphia, Tronto, 1982, p. 738.
3) T. W. Barrowcliffe and D. P. Thomas, "Haemostasis and Thrombosis," ed. by A. L. Bloom and D. P. Thomas, Churchill Livingstone, Edinburgh, 1987, p. 849.
4) J. P. Miletich, C. M. Jackson and P. W. Majerus, *J. Biol. Chem.*, **253**, 6908 (1978).
5) K. Hirahara, N. Andoh, T. Matsuishi, N. Suzuki, T. Terao and M. Kurata, *Yakugaku Zasshi*, **109**, 860 (1988).
6) K. Hirahara, T. Matusishi, N. Suzuki, T. Terao and M. Kurata, *Yakugaku Zasshi*, **109**, 867 (1988).
7) E. D. Gomperts and M. L. Zucker, *Thromb. Res.*, **12**, 105 (1977).
8) K. Hirahara, Y. Etoh, T. Matusishi, N. Suzuki and M. Kurata, *Chem. Pharm. Bull.*, **37**, 692 (1989).
9) D. Stern, P. Nawroth, J. Marcum, D. Handley, W. Kisiel, R. Rosenberg and K. Stern, *J. Clin. Invest.*, **75**, 272 (1985).
10) K. Bartha, T. Kovács, I. Lérant, B. Papp, E. Csonka, K. Kolev and R. Machovich, *Thromb. Res.*, **47**, 541 (1987).
11) Y. Nemerson, *Blood*, **71**, 1 (1988).
12) D. Stern, P. Nawroth, D. Handley and W. Kisiel, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 2523 (1985).
13) J. R. Maynard, C. A. Heckman, F. A. Pitlick and Y. Nemerson, *J. Clin. Invest.*, **55**, 814 (1975).
14) P. P. Nawroth and D. M. Stern, *J. Exp. Med.*, **163**, 740 (1986).
15) W. Straub and F. Duckert, *Thromb. Diath. Haemorrh*, **5**, 402 (1961).
16) S. A. Silverberg, Y. Nemerson and M. Zur, *J. Biol. Chem.*, **252**, 8481 (1977).
17) J. Jesty, *Arch. Biochem. Biophys.*, **185**, 165 (1978).
18) S. Kondo and W. Kisiel, *Thromb. Res.*, **46**, 325 (1987).
19) F. A. Ofosu, A. L. Cerskus, J. Hirsh, L. M. Smith, G. J. Modi and M. A. Blajchman, *Br. J. Haematol.*, **57**, 229 (1984).
20) I. Danishefsky, A. Zweden and B. L. Slomiany, *J. Biol. Chem.*, **253**, 32 (1978).
21) H. Nagasawa, B. K. Kim, M. Steiner and M. G. Baldini, *Thromb. Haemostas.*, **47**, 157 (1982).
22) A. Hensen and E. A. Loeliger, *Thromb. Diath. Haemorrh.*, **9** (Suppl. 1), 18 (1963).
23) Y. Nemerson and L. P. Clyne, *J. Lab. Clin. Med.*, **83**, 301 (1974).
24) J. Renart, J. Reiser and G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 3116 (1979).
25) P. Grabar and C. A. William, *Biochim. Biophys. Acta*, **10**, 193 (1953).
26) H. Nakamura and T. Sugiura, "Zoku Seikagaku Jikken Koza. Vol. 5, Men'eki Seikagaku Kenkyu Ho," ed. by T. Yamakawa and K. Imaho, Tokyo Kagaku Dojin, Tokyo, 1986, p. 40.
27) R. D. Rosenberg and P. S. Damus, *J. Biol. Chem.*, **248**, 6490 (1973).
28) F. J. Walker and C. T. Esmon, *Biochim. Biophys. Acta*, **585**, 405 (1979).
29) P. Bernfield, J. S. Nisselbaum, B. J. Berkley and P. W. Hanson, *J. Biol. Chem.*, **235**, 2852 (1960).
30) R. E. Jordan, G. M. Oosta, W. T. Gardner and R. D. Rosenberg, *J. Biol. Chem.*, **255**, 10073 (1980).
31) N. Heimburger, *Hoppe-Seyler's Z. Physiol. Chem.*, **353**, 1957 (1972).
32) L. V. M. Rao, *Thromb. Res.*, **51**, 373 (1988).
33) S. V. Rana, H. J. Reimers, M. S. Pathikonda and S. P. Bajaj, *Blood*, **71**, 289 (1988).
34) T. Kubota, K. Andoh, N. Kobayashi and T. Maekawa, *Thromb. Haemostas.*, **54**, 258 (1985).
35) M. Maki, T. Terao, T. Ikenoue, T. Kakemura, K. Sekiba, K. Shirakawa and H. Souma, *Gynecol. Obstet. Invest.*, **23**, 230 (1987).