

Anniversary Issue Contribution

Reminiscences of my contributions to *Thrombosis and Haemostasis*: Urine tissue factor and plasma plasmin inhibitor

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The 50th anniversary of the journal *Thrombosis and Haemostasis* (formerly named *Thrombosis et Diathesis Haemorrhagica*) brings to mind memories of the 50 years of my career as a clinical researcher.

In 1957 when the first issue of the journal appeared, I was a resident physician in a hospital in a rural area near Tokyo. One of my first clinical experiences was treating a patient with anticoagulant therapy for venous thrombosis. After training for three years in internal medicine, I joined a research group that specialized in thrombosis and haemostasis in Tokyo University Hospital. My first assignment was to take care of patients who were receiving oral anticoagulants. At that time no commercial tissue thromboplastin was available in Japan, and I had to prepare tissue thromboplastin for myself to carry out prothrombin time assays. I collected brains from sacrificed rabbits from other research laboratories where animal experiments were performed, homogenized them in a mortar by hand and finally transformed them into an acetone-dried powder, which was then used as tissue thromboplastin for prothrombin time assay. This experience was the real start of my career as an investigator in the field of thrombosis and haemostasis. From this time on, *Thrombosis et Diathesis Haemorrhagica*, later *Thrombosis and Haemostasis*, became one of the journals that I looked over most frequently. My first contribution to the journal was in 1966. Since then I have made 11 contributions to the journal, including seven original papers and four Letters to the Editor. Most of these contributions were concerned with two subjects, urine tissue factor and plasma plasmin inhibitor.

Urine tissue factor

In 1963, I got a chance to work in the laboratory of Dr. Kurt N. von Kaulla in Denver (University of Colorado, USA). In those days Dr. von Kaulla maintained a great interest in procoagulant activity in urine. The presence of such a procoagulant activity was noticed back in 1935 (1). When I joined his laboratory, he had already published a paper concerning urinary procoagulant in *Thrombosis et Diathesis Haemorrhagica* together with Walter

Seegers' group in Detroit (2). In this paper, they showed that urinary procoagulant needs factor V and platelets or phospholipids for its full activity. This requirement for platelets or phospholipids further aroused his interest in urinary procoagulant, and he even dreamed of its clinical application for haemophilic patients. He asked me to prepare a purified preparation of urinary procoagulant for animal experiments. I obtained a partially purified preparation of procoagulant from human urine and injected it intravenously into dogs. Its rapid injection caused generalized intravascular clotting with haemorrhage into parenchymal organs, and the dog died. When it was given very slowly, however, the dogs survived. There was a marked but transient shortening of the recalcification time and prolongation of serum prothrombin time. Vomiting, passing of loose stools, urination, prostration, and transient drop of blood pressure occurred. These side effects were probably caused by contaminating kallikrein-like materials and avoided by preheating the preparations. I left Denver to return to Japan in 1964, and the publication of the results was delayed until 1966 (3). This was my first appearance as an author in *Thrombosis et Diathesis Haemorrhagica*.

In 1967, I again joined Dr. von Kaulla's laboratory in Denver. He still maintained his interest in urinary procoagulant and asked me to characterize its properties. I obtained the partially purified procoagulant by methods including adsorption chromatography and gel filtration. The procoagulant slowly activated prothrombin in the presence of factors V, VII, and X and phospholipids or platelets. The requirement for exogenous phospholipids or platelets in prothrombin activation was more evident for the purified procoagulant than for crude procoagulant. The phosphorus content of the purified procoagulant was low as compared with that of the crude procoagulant or with tissue thromboplastin obtained from brain. The ratio of phosphorus to protein in the purified procoagulant was less than one tenth of that in brain tissue thromboplastin and less than a half of that in the crude procoagulant. These results suggested that urinary procoagulant represents the protein component of a thromboplastin-like material that is derived from the kidney or/and urinary tract. These results were published in *Thrombosis et Diathesis Haemorrhagica* in 1971 (4).

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After returning to Japan in 1972, I was more involved in doing research on plasmin inhibitor, and my interest on urinary procoagulant faded away. Six years later, however, we restarted our study on urinary procoagulant. We obtained human urinary procoagulant with higher purity than from the previous preparations. The procoagulant activity of the purified material was inhibited by concanavalin A but restored by the addition of α -methylglucosidase (5, 6). The findings indicated that the procoagulant is a glycoprotein and that a sugar moiety may be attached at or near a functionally critical domain in the molecule. The results were quite consistent with those reported on the tissue factor derived from human brain (7). The studies as mentioned above suggested that urinary procoagulant consists of a protein portion and lipids. We were able to separate its protein portion (apoprotein) from the lipids by introducing hydrophobic chromatography (6). The apoprotein itself did not exhibit any procoagulant activity, but upon reassociation with lipids it accelerated factor VIIa-catalyzed activation of factor X by forming a stoichiometric complex with the enzyme. The findings elucidated the mechanism of the procoagulant activity and strongly suggested that urinary procoagulant was similar or identical to tissue factor. The identity of urinary procoagulant with tissue factor was finally confirmed by the finding that the procoagulant activity in human urine was completely inhibited by a monoclonal antibody to human tissue factor (8, 9).

Since the urinary procoagulant was found to be identical to tissue factor, we used monoclonal antibodies to human tissue factor to study procoagulant functions in urine (9, 10) and also to examine tissue factor antigen in plasma (10). Ultracentrifugation and subsequent immunoblot analysis after treatment with the detergent Triton X-100 indicated that human plasma and urine contained 50,000 \times g or 100,000 \times g sedimental and non-sedimental forms of tissue factor (9, 10). Sedimental forms may well be lipoprotein- or membrane (phospholipid)-associated forms. Pre-

viously, procoagulant activity in urine was shown to be associated with subcellular particles (11). Western blotting of urine tissue factor revealed a major band of 28 kDa plus more than 10 minor bands, including protein bands of various molecular sizes from less than 14 kDa up to 100 kDa (9). Sedimental forms (re-suspended pellet of ultracentrifugation) exhibited a major band of 46–49 kDa and a minor band of 100 kDa (9, 10). Plasma tissue factor exhibited 29 kDa, 135 kDa, and a large aggregate with a molecular mass of more than 200 kDa (10). The diversity in molecular size may represent various degrees of association of tissue factor with lipids as well as contents of sugar moieties and/or its proteolytic degradation. These differences may also depend on the variety of the cellular origin of tissue factor production.

To identify the cells producing and secreting procoagulant into urine, we prepared antiserum by immunizing rabbits with purified urinary procoagulant. The antiserum harvested showed a single line against both purified urinary procoagulant and concentrated urine when examined by counter-immunoelectrophoresis (5). The antiserum did not react with whole serum, but it neutralized the procoagulant activity in purified urinary procoagulant and normal urine (5, 12). By immunofluorescence and immunoenzymatic techniques with this monospecific antibody we localized urinary procoagulant in the kidney to tubular epithelial cells of the loop of Henle and distal convoluted tubules (12) (Fig. 1). Glomeruli, the proximal tubular cells, vascular wall, and the interstitium did not stain positive. As the picture of the fluorescent staining was beautiful, I put it in a frame and hung it on the wall of my office. The procoagulant was found prominently along the luminal and intercellular borders, suggesting that the procoagulant is associated with the plasma membrane. Its association with cell membrane is in accordance with a common property of tissue factor, which is an integral membrane protein. Lwaleed et al. found tissue factor antigen in both distal and proximal tubules but not in glomerular cells (13). Other experimental and clinical studies (14–17) supported localization of tissue factor in renal tubules. In contrast to these studies, Drake et al. reported that the tissue factor expression was found in glomeruli but not in tubules (18). Fleck et al. (19) and Bukovsky et al. (20) also localized tissue factor in the glomeruli. The absence of tissue factor antigen in vascular endothelial cells is a common finding in all these studies. Tissue factor activity in urine was increased in patients with bladder cancer (21) and prostatic cancer (22). In these patients, increased tissue factor in urine may be derived directly from cancer cells.

Recently, Lwaleed et al. reported constitutive expression and secretion of tissue factor in a human kidney proximal tubular cell line and stimulation of the excretion by *E. coli* lipopolysaccharide *in vitro* (23). The tissue factor was released in parallel with a decrease in the intracellular tissue factor levels. From these observations together with reports of increased levels of tissue factor activity in urine from patients with malignancy (breast and colorectal cancer) and inflammatory bowel diseases (8, 24, 25), they hypothesized that bioactive stimulants released from inflammatory and malignant cells into the vascular compartment were excreted via the kidney, concentrating in urine to stimulate tubular cells in transit to release tissue factor. It is less likely that the increased tissue factor levels in urine are derived from the blood, since the lipid-associated molecule is too large to pass

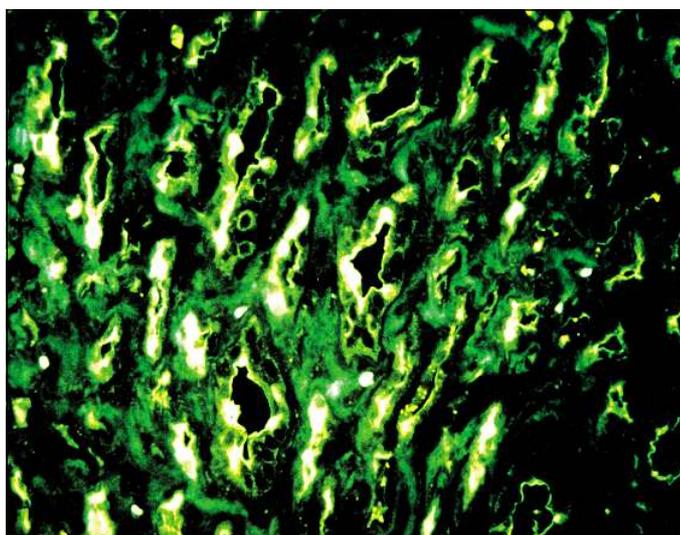


Figure 1: Immunofluorescence study showing the localization of urinary procoagulant (tissue factor) in a human kidney section. Section showing fluorescent staining in the epithelial cells of the limbs of Henle. Staining is localized more prominently on the luminal surface of the tubular cells. (For details see [12]).

through the glomerular basement membrane unless glomeruli are damaged.

In glomerulonephritis, particularly immune complex glomerulonephritis, tissue factor activity in urine is increased (26), but its source is uncertain because we found that the tissue factor antigen level in blood plasma of patients with chronic renal failure due to chronic glomerulonephritis is not elevated unless they are receiving haemodialysis (10). Interestingly, tissue factor antigen in urine decreased significantly in patients with diabetes mellitus as compared to healthy subjects (9) in spite of a significant increase of tissue factor antigen in the blood plasma (10). This suggests that an increase of tissue factor in circulating blood does not simply contribute to the level of tissue factor in urine. Probably the diabetic changes in the kidney injure epithelial cells which highly express tissue factor, resulting in the reduction of tissue factor concentrations in urine. Chronic renal failure, except for that of diabetic nephropathy, does not cause an elevation of tissue factor in blood plasma unless a patient is receiving haemodialysis, and there was no positive correlation of plasma tissue factor antigen levels with serum creatinine levels found in chronic renal failure (10), suggesting a minor role of the kidney in clearance of plasma tissue factor. Patients with microangiopathies, including polyarteritis nodosa, Wegener's granulomatosis, and diabetic microangiopathy such as retinopathy and nephropathy, have higher blood plasma levels of tissue factor than do normal healthy subjects (10). Chronic haemodialysis may also injure endothelial cells and cause an increase of tissue factor in plasma (10). Accordingly, we suggested in a letter to the editor of *Thrombosis and Haemostasis* that an increase of plasma tissue factor may reflect endothelial cell injury in the microvasculature rather than the upregulation of tissue factor expression (27), although vascular endothelial cells do not constitutively express tissue factor.

The relationship of urine tissue factor to plasma tissue factor is intriguing. Recently, a form of human tissue factor that is generated by alternative splicing was identified that lacks a transmembrane domain (28). This alternatively spliced human tissue factor (asHTF) is soluble, circulates in blood, and exhibits procoagulant activity when exposed to phospholipids. Although the presence of asHTF in urine has not been confirmed, it is possible that epithelial cells in the kidney produce asHTF and also that asHTF in blood is excreted through the kidney into urine because asHTF is smaller than full-length tissue factor.

As to a functional role of tissue factor in urine, we can affirm that it certainly facilitates haemostasis in the urinary tract. If urine lacks procoagulant activity, haemostasis at an injured inner surface of the urinary tract is probably difficult, and oozing continues because the surface is constantly rinsed by fluid (urine). Thus, the presence of tissue factor in urine is particularly beneficial in haemophilic patients. On the other hand, urine contains a powerful thrombolytic substance called urokinase. Massive bleeding into urine may form blood clots large enough to obstruct the urinary tract because of the presence of tissue factor. This risk is diminished by the presence of urokinase which dissolves blood clots through generation of plasmin.

Plasma plasmin inhibitor

Fibrin clots formed by the addition of thrombin to a mixture of fibrinogen, plasminogen, and plasminogen activator lyse spontaneously upon incubation. We called it activator-induced clot lysis. I found as early as in 1969 that this activator-induced clot lysis is efficiently inhibited by a hitherto unknown inhibitor in plasma, and the first paper describing the presence of this new inhibitor was published in 1969 in *Thrombosis et Diathesis Haemorrhagica* (29). After that I tried to isolate this new inhibitor of fibrinolysis, and finally in 1976 Moroi and I succeeded in isolating it in a pure form and characterized its properties (30). We named it α 2-plasmin inhibitor, because the inhibitor immediately neutralizes plasmin generated in the clot resulting in prolongation of activator-induced clot lysis and migrated as an α 2-globulin in immunoelectrophoresis. Although the inhibitor neutralizes various proteolytic enzymes including plasminogen activators, its main role is inhibition of plasmin (30–32). During the same period, Gallimore, Collen, as well as Müllertz and Clemmensen described the presence of a new inhibitor which later turned out to be the same as α 2-plasmin inhibitor (α 2PI) (33–35). They called the inhibitor inter- α -antiplasmin, antiplasmin, or primary plasmin inhibitor, respectively. In addition, Ulla Hedner had described an inhibitor of plasminogen activation (36). In 1994, the term plasmin inhibitor was fixed by an international cooperative committee, whereas the other names for this serine protease inhibitor were not recommended (37). I agree that plasmin inhibitor (PI) is a term that distinguishes it from plasminogen activator inhibitor (PAI) and is preferable. Accordingly, plasmin inhibitor (PI) is used as the term for the inhibitor in the rest of this article as well as in our last two papers in *Thrombosis and Haemostasis* (38, 39) and in one recent review article (40).

One month before the publication of our paper on the isolation of PI in the *Journal of Biological Chemistry* (30), the International Committee on Thrombosis and Haemostasis (predecessor of the Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis) met in Kyoto, Japan, followed by the Seminar on the Molecular Mechanism of Blood Coagulation and Fibrinolysis, sponsored by Osaka University, Osaka, Japan. In the seminar, I had the honor of speaking on the new inhibitor of fibrinolysis in front of many outstanding scientists from the US and Europe such as Birger Blombäck, John Finlayson, Nobert Heimburger, Craig Jackson, Laszlo Lorand, and Björn Wiman. Wiman expressed great interest in the strong affinity of the inhibitor to plasminogen and the efficient affinity chromatography using plasminogen as a ligand in the purification process, and asked me several questions. Wiman and Collen later utilized a modified form of this affinity chromatography and purified PI which they named antiplasmin (41).

Soon after the purification of PI, we prepared monospecific antibodies against the inhibitor and immunochemically analyzed plasma samples obtained from various patients. We concluded from these studies that PI is the most rapidly functioning inhibitor of plasmin *in vivo* (31). By immunofluorescence technique, we found the bright greenish staining for PI in cytoplasm of liver parenchymal cells (42) (Fig.2), indicating that PI is produced in the liver. This is compatible with clinical studies (43).

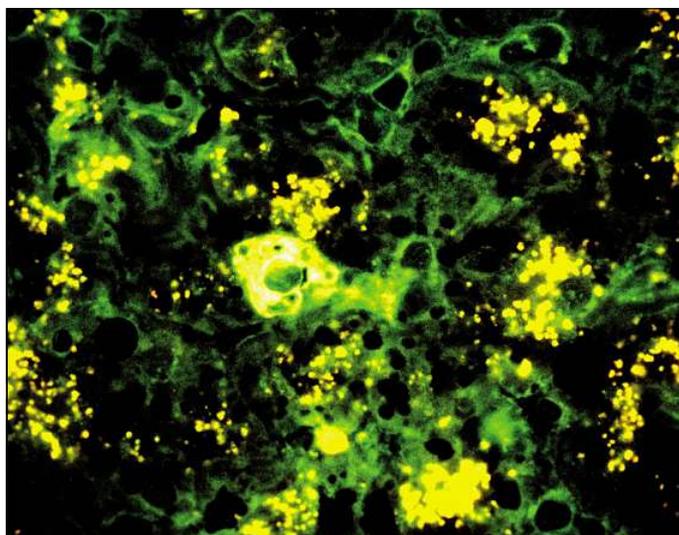


Figure 2: Immunofluorescence study showing the localization of α_2 -plasmin inhibitor in the cytoplasm of human liver parenchymal cells. In a single liver cell cytoplasm was stained leaving out nucleus and granules. Granular scattered spots of autofluorescence by lipofuscin are seen. (See also [42]).

One of the most characteristic properties of PI is its competitive inhibition of plasminogen binding to fibrin (44, 45). The binding of plasminogen to fibrin may be an initial step of endogenous fibrinolysis, and its inhibition by PI plays an important role in the retardation of endogenous fibrinolysis. ϵ -amino-caproic acid, a known inhibitor of plasminogen binding to fibrin, inhibits PI binding to plasminogen, indicating that the site in the plasminogen molecule which binds PI is the same site by which plasminogen binds to fibrin. We presented these results in *Thrombosis and Haemostasis* in 1978 (45), being my first contribution concerning PI to this journal under its new name.

Another important function of PI is its binding to fibrin. When a fibrin clot is formed, a certain part of PI rapidly binds to fibrin. The binding is a covalent interaction (cross-linking) catalyzed by activated factor XIII and the binding renders the fibrin clot resistant to fibrinolysis (46, 47). An increase of the resistance to fibrinolysis by cross-linking of PI to fibrin may be more significant when the fibrin clot is retracted by platelets (47, 48). Factor XIIIa-catalyzed cross-linking to fibrin is a characteristic property of PI among factors involved in haemostasis. Thus the cross-linking of PI to the fibrin α -chain (49, 50) in addition to inhibition of plasmin binding to fibrin exceeds the concept of a protease inhibitor and designates PI as an inhibitor of fibrinolysis rather than a mere plasmin inhibitor.

The real physiological significance of PI was revealed by the discovery of the first case of its congenital deficiency in 1979 (51), soon after the isolation of PI from human plasma. The patient was a 16-year-old boy living on an island located in Southwest Japan, who had been treated as a peculiar type of haemophiloid without an exact diagnosis for over 10 years. He had been suffering from severe haemorrhagic tendency since his early childhood. The patient was found to be a homozygote completely lacking PI in his blood. The most remarkable laboratory finding in the patient was spontaneous clot lysis (51, 52). When the patient's plasma clotted, plasminogen

and plasminogen activator are bound to fibrin, and plasminogen activation takes place on the fibrin surface (53), causing complete lysis of fibrin by the generated plasmin. Thus, the endogenous fibrinolytic process proceeds without hindrance and haemostatic plugs once formed are dissolved prematurely before the restoration of injured vessels, resulting in a haemorrhagic tendency.

We analyzed the molecular basis of PI deficiency in four different families. One family had a point mutation in an intron, resulting in very short transcripts and non-production of the mature protein (38). The other three families have a deletion or insertion in an exon coding for PI (39, 54–56). The mutant PIs of these three families were found to be degraded within cells, while their transport was retarded in the intracellular secretory pathway so that they are not secreted into the plasma. We also found that a similar impairment of intracellular transport of mutant proteins occurs in protein C deficiency (57). Therefore, we suggested in a letter to the editor of *Thrombosis and Haemostasis* that impaired intracellular transport may be one of the prevalent causes for the deficiencies of factors involved in coagulation and fibrinolysis (58).

Recently, I described in detail anecdotally and vividly how we discovered the inhibitor and its congenital deficiency as a historical sketch in the *Journal of Thrombosis and Haemostasis* (59), and the story was replenished by M. J. Gallimore (60).

In a recent review, I described the development of our knowledge about plasmin inhibitor from the beginning to the present, including recent advances (40). This is a comprehensive review covering most of the important references from 1971 to the middle of 2004; but here I must refer to the latest developments, which were not dealt with in the review and may have great impact on future investigations of PI. In 1989, Sumi et al. expressed human PI in mammalian cells and found that a PI that has an additional 12 amino acid sequence at the NH₂-terminus with an NH₂-terminal Met (Met-PI) was present in addition to a PI with an NH₂-terminal Asn (Asn-PI) (61). They postulated that Met-PI might be pro-PI. In 1993, however, Bangert et al. found that Met-PI is present in plasma as a mature protein in addition to Asn-PI (62). Subsequently, Koyama et al. reported that PI produced and secreted by human liver cells is totally Met-PI but is then converted to Asn-PI in blood plasma (63), indicating that Met-PI is a mature protein and Asn-PI is its proteolytic derivative. Met-PI and Asn-PI have nearly the same inhibitory activity on plasmin (61, 64), but Met-PI has remarkably less FXIIIa-catalyzed cross-linking activity than Asn-PI (61, 64). Thus, the conversion of Met-PI to Asn-PI in plasma during circulation, by releasing the 12-amino acid peptide from the NH₂-terminus, renders the fibrin clot formed more resistant to fibrinolysis (61, 63, 64). Recently, Lee et al. identified a proteinase in plasma that is responsible for the proteolytic cleavage of the Pro12-Asn13 bond of Met-PI to yield Asn-PI (64); and they further obtained evidence to show that the responsible enzyme is a soluble derivative of a cell-surface proteinase called fibroblast activation protein or seprase (65). From these important findings, one could speculate about the possibility of reducing the amount of PI bound to fibrin by blocking the activity of this protease, so that thrombolysis would be facilitated. Interestingly, they also found that a single nucleotide polymorphism affects the conversion of Met-PI to Asn-PI, and may play a significant role in governing the long-term deposition and removal of intravascular fibrin (66).

Other subjects

In addition to the subjects described above, we made two contributions to *Thrombosis and Haemostasis* concerning different subjects. One concerns protein C and the other thrombomodulin. In the former contribution (67), we reported the effects of infusion of purified protein C and activated protein C on coagulation and fibrinolysis of normal volunteers. This study developed into the clinical use of activated protein C in disseminated intravascular coagulation (68). In the latter contribution to *Thrombosis and Haemostasis* (69), we emphasized our previous findings that thrombomodulin levels in plasma were specifically increased in systemic small vessel vasculitis (70, 71).

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Concluding remarks

As described above, we made a total of 11 contributions to *Thrombosis et Diathesis Haemorrhagica* or *Thrombosis and Haemostasis*. We are proud of these contributions, and we hope that *Thrombosis and Haemostasis* continues to be one of the most important journals in medical sciences and an indispensable resource for researchers in our field.

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