Urinary Protein C Inhibitor as a Therapeutic Agent to Disseminated Intravascular Coagulation (DIC): A Comparison with Low Molecular Weight Heparin in Rats with Lipopolysaccharide-Induced DIC

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We compared urinary protein C inhibitor (uPCI) with low molecular weight heparin (LMWH) in terms of the effect on the pathophysiology of disseminated intravascular coagulation (DIC), such as hypercoagulation, induction of secondary fibrinolysis and organ failure, using lipopolysaccharide (LPS)-induced DIC in rats. The uPCI (0.5 and 1.0 mg/kg) administration significantly inhibited both the decrease in fibrinogen level and the increase in fibrin/fibrinogen degradation products (FDP) level, and the effects compared favorably with those of LMWH (100 and 200 IU/kg). Both uPCI (0.5 and 1.0 mg/kg) and a low dose of LMWH also inhibited the increases in the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), thrombin, and plasma kallikrein equally, but a high dose of LMWH did not inhibit the changes in those parameters. Furthermore, uPCI dose-dependently prevented the prolongation of activated partial thromboplastin time (APTT), while LMWH excessively prolonged APTT at a high dose.

These results suggest that the preventive effect of uPCI on the pathophysiology of DIC compares favorably with that of LMWH, including the lack of a hemorrhagic reaction in contrast to LMWH.

Key words protein C inhibitor; disseminated intravascular coagulation; low molecular weight heparin

Disseminated intravascular coagulation (DIC) occurs in several diseases such as sepsis and cancer, and is a systemic hemorrhagic syndrome which results from the consumption of coagulation factors for the formation of multiple thrombi in the systemic microvessels; it is associated with multiple organ failure.¹⁻³⁾ Especially, in the pathophysiology of DIC in sepsis, the protein C anticoagulant pathway is downregulated by a significant decrease in the thrombin-thrombomodulin complex, accompanied by a sustained decline in the expression of thrombomodulin on endothelial cells.⁴⁾ In addition, the blood coagulation system is activated by an increase in tissue factor expression on endothelial cells and by the expression of tissue factor on small vesicles released from stimulated endothelial cells.^{4,5)} As a result, the condition of the blood coagulation system shifts to hypercoagulation, and multiple thrombi are formed in the systemic microvessels. Therefore, for DIC therapy, unfractionated heparin (heparin) and low molecular weight heparin (LMWH) are used as anticoagulant agents.

Protein C inhibitor (PCI) has been known as an inhibitor of the protein C anticoagulant pathway under normal conditions. PCI suppresses this pathway through the inhibition of activated protein C (APC) itself and of protein C activation by the thrombin-thrombomodulin complex.^{6,7)} However, PCI inhibits not only APC but also procoagulant proteases such as thrombin, factor Xa, factor XIa and plasma kallikrein,⁸⁻¹³⁾ and it is also known as a serine protease inhibitor.^{14,15)} Therefore, it has been thought that PCI possibly possesses a broad spectrum of protease regulation. We hypothesized that PCI possibly acts as an inhibitor to procoagulant proteases under hypercoagulation of DIC, accompanied by a decrease in thrombomodulin expression on endothelial cells, and the effect of urinary PCI (uPCI) on DIC has been investigated using LPS-induced experimental DIC in rats.¹⁶⁾ When uPCI was intravenously administered into the DIC model at high concentrations (20—40 times higher than the normal plasma

level), drastic changes in the parameters of blood coagulation, fibrinolysis and organ failure were significantly prevented. Thus, we suggested that uPCI improved the pathophysiology of DIC (*e.g.* hypercoagulation, organ failure) through inhibition of the activities of thrombin and plasma kallikrein.¹⁶⁾

In the present study, to evaluate the value of uPCI as an agent for the clinical treatment of DIC, we compared uPCI with LMWH in terms of the effect on the pathophysiology of the experimental DIC induced by LPS.

MATERIALS AND METHODS

Animals Male SD rats were obtained from Charles River Japan, Inc., Kanagawa, Japan. The animals were fed standard laboratory diet and water *ad libitum*.

Reagents A PCI antigen ELISA assay kit was purchased from Technoclone, Vienna, Austria. Low molecular weight heparin (from porcine intestinal mucosa) was purchased from Serbio Laboratory, Gannevilliers, France. An APTT test kit (APTT-test Wako), PT test kit (PT-test Wako) and fibrinogen test kit (Fibrinogen-test Wako) were obtained from Wako Pure Chemical Industries, Osaka, Japan. An FDP test kit (FDP latex agglutination test) was from Teikoku Hormone Mfg., Tokyo, Japan. Complete blood multi-rotor V-4 and synthetic substrates S-2238 (for thrombin), S-2302 (for plasma kallikrein) and S-2366 (for activated protein C) were from Daiichi Pure Chemicals, Tokyo, Japan. Lipopolysaccharide (*E. coli* 0127:B8) was from Difco Laboratories, Detroit, MI, U.S.A. Chelate-Sepharose, concanavalin A-Sepharose, heparin-Sepharose CL-6B and Sephadex G-100 were from Amersham Pharmacia Biotech, Uppsala, Sweden. All other reagents used were obtained from commercial sources.

Purification of Urinary Protein C Inhibitor uPCI was purified from freshly voided human urine as described previously.¹⁶⁾ uPCI was isolated by sequential use of affinity chro-

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matography on zinc chelate-Sepharose, concanavalin A-Sepharose, heparin-Sepharose, and gel-filtration on Sephadex G-100. Molecular weight and the purity of the purified uPCI were 54 kDa and over 95%, respectively, as estimated by SDS-PAGE.¹⁶⁾ The concentration of uPCI was determined by the PCI antigen ELISA assay kit. IC₅₀ values of uPCI for 10 nM human APC, thrombin and plasma kallikrein were measured as described previously,¹⁶⁾ and were 250, 150 and 100 nM, respectively, in the absence of heparin, and 30, 40 and 40 nM, respectively, in the presence of heparin (5 units/ml).

Animal Experiments A schematic of the experimental protocol is shown in Fig. 1. Male SD rats weighing 170–200 g were anesthetized with pentobarbital (50 mg/kg), then LPS was continuously infused for up to 60 min *via* the tail vein at a dose of 50 mg/kg. We recently demonstrated that the blood coagulation system and secondary fibrinolysis were markedly activated between 60 and 120 min after the end of LPS infusion (50 mg/kg).¹⁶⁾ Therefore, in the animal experiments, we used the rats at 60 min after the end of LPS infusion as a DIC model. uPCI (0.5 or 1.0 mg/5 ml/kg) or LMWH (100 or 200 IU/5 ml/kg), which were dissolved in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, were continuously administered for 60 min *via* the left femoral vein of rats with LPS-induced DIC. Rats in the control group were given the vehicle (5 ml/kg) instead of the agents. Rats in the normal group were given the vehicle instead of LPS and the agents. Seven animals were used in the normal and control groups, and 5 animals were used in the agents administration groups. Immediately prior to and 30 and 60 min after the beginning of uPCI, LMWH or vehicle administration, blood samples were withdrawn from the right jugular vein and used for the determination of platelet count (150 μ l, collected with EDTA), plasma fibrinogen level (100 μ l, collected with 1/10th volume of 0.13 M sodium citrate) and FDP level (100 μ l, collected with coagulant which was enclosed in FDP test kit). At the end of the experiment, 150 μ l of blood sample for the measurement of AST and ALT was collected with heparin from the right jugular vein, and 2 ml of blood sample was collected from the inferior vena cava vein of each rat into a syringe containing 1/10th volume of 0.13 M sodium citrate, then the citrate blood sample was centrifuged at 2000 $\times g$ for 10 min at 4 °C to obtain plasma samples. These were used for the determination of APTT, PT, and the levels of thrombin and plasma kallikrein.

Determination of Hematological Parameters Platelet count was determined using a hemocounter (Microcell-counter F-800, Sysmex, Kobe, Japan). APTT, PT and fibrinogen level were determined by a coagulometer (KC 1A, Amelung, Lemgo, Germany) using the APTT, PT and fibrinogen test kits, respectively. AST and ALT were measured by an analyzer of blood biochemistry (ABAXIS EA, Daiichi Pure Chemicals) using the complete blood multi-rotor V-4. FDP level was determined by an FDP test kit.

Synthetic Substrate Assays for Thrombin and Plasma Kallikrein The levels of thrombin and plasma kallikrein in the plasma samples were determined in 96-well plates at 37 °C as follows: 10 μ l of plasma was mixed with 40 μ l of 10 mM Tris-HCl buffer, pH 8.4, and added to 50 μ l of 2 mM substrate (S-2238 or S-2302). After the mixture was incubated for 30 min at 37 °C, 60 μ l of 50% acetic acid was

Fig. 1. Schematic of Experimental Protocol

Male SD rats (170–200 g) were anesthetized, and LPS (50 mg/kg) was continuously infused for up to 60 min *via* the tail vein. The rats were left alone for 60 min after the end of LPS infusion to establish the DIC model, then uPCI or LMWH was continuously administered *via* the left femoral vein for 60 min. The vehicle was administered as a control in the same manner. Blood samples were drawn at the indicated time and used for the measurement of the parameters. Duration of the experimental protocol was 180 min. Values are time points during the experiment: -120 and -60, 120 and 60 min before the beginning of each agent administration, respectively; Pre, prior to each agent administration; 30 and 60, 30 and 60 min after the beginning of each agent administration, respectively.

added to stop the reaction. The absorbance at 405 nm was measured by a microplate reader (THERMOMax Kinetic Microplate Reader, Molecular Devices, Menlo Park, CA, U.S.A.).

Statistical Analysis All data are expressed as the mean $\pm$ S.D. The variances among groups were analyzed using Bartlett's test, then the statistical significance of differences between the control group and each treatment group was analyzed by Dunnett's test (for parametric data) or Sheffe's test (for nonparametric data). Comparisons between the control group and the normal group were made using Student's *t*-test. *p* values less than 0.05 were considered to be statistically significant.

RESULTS

Effects on Changes in Platelet Count, Fibrinogen Level and FDP Level in Rats with LPS-Induced DIC As shown in Fig. 2, in the control group, decreases in the platelet count and fibrinogen level and an increase in FDP level were observed with LPS infusion. In the normal group, platelet count, fibrinogen level and FDP level were not changed as compared with the values of normal condition, and the difference in values between the control group and the normal group at each time was statistically significant ($p < 0.05$) (data not shown). The uPCI (0.5 and 1.0 mg/kg) administration significantly inhibited both the decrease in fibrinogen level and the increase in FDP level in the experimental DIC (Figs. 2B and 2C). The inhibitory effect of uPCI on the decrease in fibrinogen level was as potent as that of LMWH (100 and 200 IU/kg), and its effect on the increase in FDP level was more potent than that of LMWH. Although uPCI did not reduce the decrease in platelet count in the experimental DIC, LMWH significantly reduced it (Fig. 2A).

Effects on Changes in APTT and PT in Rats with LPS-Induced DIC The effects of uPCI and LMWH on changes in APTT and PT in the experimental DIC are shown in Table 1. In the control group, APTT and PT were significantly prolonged as compared with the normal group. At all doses, uPCI significantly prevented the prolongation of APTT and PT. In contrast, although LMWH tended to dose-dependently prevent the prolongation of PT, excessive prolongation of APTT was observed at a dose of 200 IU/kg.

Effects on Changes in AST and ALT Levels in Rats with LPS-Induced DIC The effects of uPCI and LMWH

Fig. 2. Effects of uPCI and LMWH on LPS-Induced DIC with Regard to Changes in Platelet Count (A), Fibrinogen Level (B) and FDP Level (C)

Pre, Δ30 and Δ60 represent time points during the experiment: Pre, prior to administration of each agent; Δ30, 30 min after the beginning of each agent administration; Δ60, 60 min after the beginning of each agent administration. Platelet count and fibrinogen levels are expressed as a % relative to the value prior to the beginning of each agent administration. Each column represents the mean ± S.D. * $p < 0.05$; ** $p < 0.01$, significant difference from control group at each time (Dunnett's test).

Table 1. Effects of uPCI and LMWH on Changes in APTT and PT in Rats with LPS-Induced DIC

	APTT (s)	PT (s)
Normal	35.4 ± 1.5	12.6 ± 0.4
Control	137.5 ± 47.2 ^{##}	32.3 ± 5.8 ^{##}
uPCI 0.5 mg/kg	80.6 ± 7.9*	21.1 ± 1.3**
1.0 mg/kg	61.7 ± 3.2**	24.1 ± 0.4**
LMWH 100 IU/kg	100.0 ± 16.4	26.6 ± 3.9
200 IU/kg	>300**	25.3 ± 3.9*

Values are the mean ± S.D. * $p < 0.05$; ** $p < 0.01$, significant difference from control group (Dunnett's test). ^{##} $p < 0.01$, significant difference between normal group and control group (Student's *t*-test).

on changes in AST and ALT levels in the experimental DIC are shown in Table 2. In the control group, the AST level and ALT level were increased 6 and 4 times as compared with those of the normal group, respectively. Both uPCI (0.5 and 1.0 mg/kg) and LMWH (100 IU/kg) administrations significantly inhibited the increases in AST and ALT levels equally, but 200 IU/kg of LMWH administration did not inhibit the increases in these levels.

Effects on Changes in Thrombin and Plasma Kallikrein Levels in Rats with LPS-Induced DIC The effects of uPCI and LMWH on changes in thrombin and plasma kallikrein levels in the experimental DIC are shown in Table 3. In comparison with the normal group, thrombin and plasma kallikrein levels observed in the control group were significantly increased. The uPCI (0.5 and 1.0 mg/kg) administration significantly inhibited the increases in thrombin and

Table 2. Effects of uPCI and LMWH on Changes in Levels of AST and ALT in Rats with LPS-Induced DIC

	AST (U/L)	ALT (U/L)
Normal	58 ± 13	19 ± 7
Control	337 ± 76 ^{##}	78 ± 24 ^{##}
uPCI 0.5 mg/kg	154 ± 41**	29 ± 11**
1.0 mg/kg	186 ± 37**	30 ± 3**
LMWH 100 IU/kg	179 ± 10**	21 ± 2**
200 IU/kg	254 ± 60	60 ± 25

Values are the mean ± S.D. * $p < 0.05$; ** $p < 0.01$, significant difference from control group (Dunnett's test). ^{##} $p < 0.01$, significant difference between normal group and control group (Student's *t*-test).

Table 3. Effects of uPCI and LMWH on Changes in Levels of Thrombin and Plasma Kallikrein in Rats with LPS-Induced DIC

	Thrombin (U/L)	Plasma Kallikrein (U/L)
Normal	44 ± 9	86 ± 16
Control	96 ± 26 ^{##}	166 ± 34 ^{##}
uPCI 0.5 mg/kg	60 ± 22*	116 ± 20*
1.0 mg/kg	52 ± 10*	125 ± 22*
LMWH 100 IU/kg	37 ± 9**	85 ± 17*
200 IU/kg	73 ± 29	160 ± 29

Values are the mean ± S.D. * $p < 0.05$; ** $p < 0.01$, significant difference from control group (Dunnett's test). ^{##} $p < 0.01$, significant difference between normal group and control group (Student's *t*-test).

plasma kallikrein levels, and these effects were equal to LMWH at a dose of 100 IU/kg. However, LMWH at a dose of 200 IU/kg did not inhibit the increases in these levels.

DISCUSSION

In the pathophysiology of DIC in sepsis, the protein C anticoagulant pathway is downregulated, and the blood coagulation system is activated.⁴⁾ As a result, the condition of this system shifts to hypercoagulation, and multiple thrombi are formed in systemic microvessels. Therefore, anticoagulant therapy using such anticoagulant agents as heparin and LMWH is a useful treatment for DIC. Although clinical use of heparin is limited by the major side effect of hemorrhage,^{17,18)} LMWH possesses an anticoagulant activity as potent as that of heparin and has lower risk of hemorrhage compared with an equivalent dose of heparin.^{19,20)} These findings demonstrate that LMWH is a more useful agent for the clinical treatment of DIC.

Recently, we have demonstrated that uPCI improves hypercoagulation, the induction of secondary fibrinolysis and organ failure in experimental DIC induced by LPS.¹⁶⁾ However, the value of uPCI as an agent for the clinical treatment of DIC is unknown. Therefore, in the present study we compared uPCI with LMWH in terms of effect on the pathophysiology of DIC using LPS-induced DIC in rats.

As shown in Fig. 2, the inhibitory effect of uPCI on the decrease in fibrinogen level was as potent as that of LMWH, but the inhibitory effect of uPCI on the increase in FDP level was more potent than that of LMWH. We have recently reported that uPCI may prevent fibrin formation through the inhibition of thrombin and plasma kallikrein activities. Moreover, uPCI may also suppress the induction of secondary fibrinolysis through the inhibition of fibrin formation and the inhibition of urokinase (two-chain urokinase-type plasminogen activator) activity.¹⁶⁾ Urokinase indirectly induces fibrinogen degradation, rather than clot lysis, because the activation of plasminogen by urokinase is systemic and independent of fibrin.²¹⁾ In contrast, LMWH is known to inhibit fibrin formation through both the enhancement of antithrombin III (ATIII) inhibitory action on factor Xa²²⁾ and the release of tissue factor pathway inhibitor (TFPI), an inhibitor to factor Xa and tissue factor/factor VIIa complex, from intravascular pools.^{23,24)} The suppression of secondary fibrinolysis by LMWH has been thought to be the result of the inhibition of fibrin formation.²⁵⁾ However, LMWH is also known to stimulate the conversion of pro-urokinase (single-chain urokinase-type plasminogen activator) into urokinase by plasmin.²⁶⁾ Supported by these reports, our results suggest that the difference between uPCI and LMWH in terms of effect on the induction of secondary fibrinolysis accompanied by hypercoagulation may be due to the difference in effects of each agent on urokinase activity.

In the experimental DIC induced by LPS, although uPCI significantly prevented the prolongation of APTT dose-dependently, LMWH exerted further prolongation of APTT at a high dose. PCI possesses a broad spectrum of inhibition of procoagulant proteases such as plasma kallikrein, factor XIa, factor Xa and thrombin,¹²⁾ so uPCI may have suppressed the consumption of coagulation factors, downstream and upstream of factor Xa during the coagulation cascade, under hypercoagulation in this experiment. On the other hand, LMWH mainly inhibits factor Xa activity by catalyzing the ATIII- and TFPI-dependent inhibition, as mentioned above, so the consumption of coagulation factors, downstream of

factor Xa, may have been inhibited by LMWH. This difference in their mechanisms of action during the coagulation cascade is thought to be the cause of the difference between uPCI and LMWH in terms of effect on the prolongation of APTT accompanied by hypercoagulation. Moreover, the anticoagulant effect of uPCI in the treatment of DIC does not seem to be accompanied by the risk for hemorrhage, in contrast to LMWH, because uPCI administration prevented the prolongation of APTT, correlated with a risk of hemorrhage, in this experiment.

Furthermore, both uPCI (0.5 and 1.0 mg/kg) and a low dose of LMWH significantly suppressed the increases in the levels of AST, ALT, thrombin and plasma kallikrein in this experimental DIC induced by LPS. On the other hand, a high dose of LMWH did not inhibit the increases in those levels. Both thrombin and plasma kallikrein are known to act not only as procoagulant proteases but also as a chemotactic factor to phagocytes such as neutrophils and monocytes, and organ failure is spurred by these procoagulant enzymes through the activation of the phagocytes.²⁷⁾ The highly selective synthetic inhibitor of plasma kallikrein (PKSI-527) suppressed the increases in AST and ALT levels in LPS-induced DIC.²⁸⁾ Therefore, our results suggest that both uPCI and a low dose of LMWH prevent organ failure through the inhibition of activities of thrombin and plasma kallikrein in the pathophysiology of DIC.

In summary, the present study indicates that the preventive effect of uPCI on the pathophysiology of LPS-induced DIC compares favorably with that of LMWH. Furthermore, this effect is not accompanied by the risk for hemorrhage, which is the undesirable side effect of LMWH. Therefore, uPCI may be a more useful agent for the clinical treatment of DIC compared with LMWH.

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