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Author(s)	Hayakawa, Mineji; Gando, Satoshi; Ieko, Masahiro; Honma, Yoshinori; Homma, Taeko; Yanagida, Yuichiro; Kubota, Nobuhiko; Uegaki, Shinji; Sawamura, Atsushi; Asakura, Hidesaku
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Title

Massive amounts of tissue factor induce fibrinogenolysis without tissue hypoperfusion in rats

Running head

Massive tissue factor induce fibrinogenolysis

Authors

Mineji Hayakawa ¹	Satoshi Gando ¹	Masahiro Ieko ²
Yoshinori Honma ¹	Taeko Homma ¹	Yuichiro Yanagida ¹
Nobuhiko Kubota ¹	Shinji Uegaki ¹	Atsushi Sawamura ¹
Hidesaku Asakura ³		

Institution

¹ Division of Acute and Critical Care Medicine, Department of Anesthesiology and Critical Care Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan

² Department of Internal Medicine, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-gun, Japan

³ Department of Internal Medicine (III), Kanazawa University School of Medicine, Kanazawa, Japan

Corresponding author

Mineji Hayakawa, MD, PhD
 Emergency and Critical Care Center, Hokkaido University Hospital,
 N14W5, Kita-ku, Sapporo 060-8648 Japan
 TEL +81-11-706-7377 / FAX +81-11-706-7378
 e-mail mineji@dream.com

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ABSTRACT — Trauma-induced tissue factor (TF) release into the systemic circulation is considered to play an important role in the development of disseminated intravascular coagulation (DIC) immediately after severe trauma. However, the relationship between TF and hyper-fibrinolysis, especially fibrinogenolysis, has been unclear. A total 18 rats were divided into three groups. (a) The control group was infused with normal saline, (b) the low dose group was infused with 4 U/kg TF, and (c) the high dose group was infused with 16 U/kg TF. Arterial blood was drawn immediately and two and four hours after the start of TF infusion. At each sampling point, arterial blood gases, platelet counts, and coagulation variables were measured. The fibrinogen degradation products (FgDP) were evaluated by a Western blot analysis. Hypotension, hypoxemia and lactic acidosis were not observed in any of the three groups. In proportion to the doses of TF, the platelet counts, coagulation and fibrinolysis variables deteriorated in line with DIC. The alpha 2-plasmin inhibitor levels significantly decreased in the high dose group compared to the other groups. The amounts of FgDP increased in proportion to the doses of TF. The plasmin-alpha 2-plasmin inhibitor complex level in the high dose group increased more than that of the other groups. In conclusions, TF can induce DIC associated with fibrinolysis and

DIC: disseminated intravascular coagulation
FgDP: fibrinogen degradation products
TF: tissue factor

fibrinogenolysis without tissue hypoperfusion. The decrease in the alpha 2-plasmin inhibitor level and the significant increase in the plasmin level may be the two main factors underlying the pathogenesis of hyper-fibrin(ogen)olysis after TF administration.

Keywords

Fibrin Fibrinogen Degradation Products

Fibrinolysin

Fibrinolysis

Multiple Trauma

Thromboplastin

INTRODUCTION

Acute coagulopathy after trauma is a complex disorder induced by multiple factors, which include hemodilution, hypothermia, acidosis, and trauma-induced coagulopathy (1-3). We have previously reported that the trauma-induced coagulopathy was due to disseminated intravascular coagulation (DIC) with the fibrinolytic phenotype (1,4).

DIC is classified as either the fibrinolytic phenotype (marked bleeding type) or the thrombotic phenotype (organ failure type) (1,5). Acute promyelocytic leukemia and obstetric calamities are other representative causes of DIC with the fibrinolytic phenotype, while the usual cause of DIC with the thrombotic phenotype is sepsis (1,5).

Brohi and colleagues argued that the trauma-induced coagulopathy is considered to be distinct from DIC, and suggested that such coagulopathy should be called Acute Coagulopathy of Trauma-Shock (ACoTS) (2). Recently, based on the results of thrombelastography, Kashuk and colleagues reported that hyper-fibrinolysis was observed in patients with trauma-induced coagulopathy, and indicated that the hyper-fibrinolysis was integral in the

ACoTS: Acute Coagulopathy of Trauma-Shock
DIC: disseminated intravascular coagulation

pathogenesis of this trauma-induced coagulopathy (3). Although acute coagulopathy after trauma sometimes occurs without hypoperfusion (1-3), hypoperfusion and hyper-fibrinolysis also affect the pathophysiology of this condition (1,2,4,6-8).

Tissue trauma and the resulting massive tissue factor (TF) release play an important role in the development of DIC with the fibrinolytic phenotype immediately after severe trauma (1,2,9-11). In several clinical reports, an elevation of the TF levels in the systemic circulation was observed in trauma patients with DIC beginning just after admission and during the next several days (11,12). This massive tissue factor release induces the activation of systemic coagulation, thrombin generation, and consumption coagulopathy (1,2,9-11).

Various experimental models of traumatic coagulopathy have been reported (13,14). An Educational Initiative on Critical Bleeding in Trauma group recommended the TF-induced DIC model as one of the best experimental models of trauma-induced coagulopathy (13,15). Asakura and colleagues (15) reported the elevation of D-dimer levels and consumption coagulopathy in the TF-induced DIC model. However, the relationship between TF and hyper-fibrinolysis, especially fibrinogenolysis, has not yet been elucidated. In the

TF: tissue factor

present study, we attempted to clarify the effects of TF administration on both coagulation and fibrin(ogen)olysis in a rat model of TF-induced DIC.

MATERIALS AND METHODS

All animals were housed and treated according to the Standards of Animal Experiments of Hokkaido University. All of our experiments were approved by the Institutional Ethical Review Board at Hokkaido University.

Animals

Male Wistar S/T rats, age 9 weeks, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were acclimated for a minimum of two days in our animal breeding quarters before starting the experiments. The breeding quarters were maintained at a constant temperature of 20°C, and the animals were fed a standard diet and given free access to water.

Experimental procedures

The rats were anaesthetized intraperitoneally with 30 mg/kg pentobarbital (Somnopenyl, Kyoritsu Seiyaku Corporation, Tokyo, Japan), and restrained in a supine position. After a small incision was made, the left femoral artery was catheterized with a 24 gauge SURFLO (Terumo, Tokyo, Japan) catheter to allow for monitoring of the mean arterial pressure and arterial blood sampling. The mean arterial pressure was monitored with a TruWave Disposable Pressure Transducer (Edwards Lifesciences, CA, USA) and a Viridia component monitoring system (Hewlett-Packard, CA, USA). To keep the arterial catheter patency, normal saline was constantly infused at 1 ml/hr. The right jugular vein was also catheterized using a silicone micro-tube (external diameter: 1 mm) for the administration of TF (Thromboplastin C pulse, Sysmex, Kobe, Japan). The thromboplastin C pulse was made from an extract from rabbit brain. TF was freshly dissolved in normal saline before the experiment. During the experimental periods, the rectal temperatures were maintained at 37-39°C.

A total of 18 rats were randomly divided into three groups: (a) The control group was constantly infused with 5 ml of normal saline for 4 hours via the right jugular vein catheter (n = 6), (b) the low dose group was constantly infused 4 U/kg TF diluted in 5 ml of normal saline for 4 hours (n =6), which was

the same method used for a previous TF-induced DIC model (15,16), and (c) the high dose group was constantly infused 16 U/kg TF diluted in 5 ml of normal saline for 4 hours (n =6). Arterial blood (3 ml) was drawn via the femoral arterial catheter before and 2 hours after the start of TF infusion. After completing blood sampling, the same amount of normal saline (3 ml) was administered. At the end of the TF infusion (4 hours after the start of TF infusion), the rats were exsanguinated via the femoral arterial catheter under deep anaesthesia provided by the inhalation of dimethyl ether. At each of the sampling points, 1 ml of the blood was immediately put into the exclusive vacuum blood collection tube for serum fibrinogen/fibrin degradation products (FDP) (Venoject II, Terumo, Tokyo, Japan), which contained thrombin, aprotinin, and snake venom. A portion of whole blood was used for the arterial blood gas analysis. The rest of the blood samples were immediately diluted (1:9 v/v) with 4% sodium citrate. All samples were promptly centrifuged and separated. The obtained serum and plasma were frozen at -80°C until the analysis.

Measurements

The arterial blood gases and lactate levels were analyzed by an ABL 700

FDP: fibrinogen/fibrin degradation products

(Radiometer, Copenhagen, Denmark). The plasma alpha 2-plasmin inhibitor level was measured by a LPIA-NV7 instrument (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The D-dimer and FDP in serum were also measured by the LPIA-NV7. The prothrombin time and fibrinogen were measured by an ACL Top coagulation analyzer (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The level of plasmin-alpha 2-plasmin inhibitor complex (PIC) was determined using a commercial enzyme-linked immunosorbent assay kit (Rat plasmin-antiplasmin complex (PAP) ELISA Kit, Cusabio Biotech Co., Ltd., Wuhan, China).

Western blot analysis

Samples (20 μ L) from the exclusive vacuum blood collection tube for serum FDP were diluted 1:2 with SDS sample buffer without a reducing agent. The diluted samples were boiled at 100°C for 5 min, and loaded onto SDS-polyarylamide gels (4-15% Mini-PROTEAN TGX gel, Bio-Rad Laboratories, Inc., CA, USA), subjected to electrophoresis, and then were electrophoretically transferred to polyvinylidene difluoride filter membranes. After the transfer, the membranes were immersed in phosphate buffered saline (PBS) with 0.03%

PBS: phosphate buffered saline

PIC: plasmin-alpha 2-plasmin inhibitor complex

Tween 20 and 5% ECL Prime Blocking Reagent (GE Healthcare Japan, Tokyo, Japan) for 1 hour at room temperature. Next, the membranes were incubated at room temperature with the primary antibody (rabbit monoclonal antibody to fibrinopeptide A (EPR2919), Abcam, Tokyo, Japan) and 5% blocking reagent in PBS-Tween buffer for 1 hour. The membranes were washed six times with PBS-Tween buffer, and incubated at room temperature with the secondary antibody coupled to horseradish peroxidase for 1 hour. The membranes were then washed again six times with PBS-Tween buffer and twice with PBS buffer. The membrane was visualized with a chemiluminescence detection kit (ECL Advance Western Blotting Detection Kit, GE Healthcare Japan, Tokyo, Japan) and a Chemiluminescence Imaging System (Light capture II, ATTO, Tokyo, Japan). The captured images were analyzed by the Cool Saver analyzer (ATTO, Tokyo, Japan). A positive control for the fibrinogen degradation products (FgDP) was made as follows: Human fibrinogen solution (Human Fibrinogen 1, Enzyme Research Laboratories, South Bend, USA) was diluted to 10 mg/ml with 0.05 M Tris-buffer. Plasmin from human plasma (P-1867, Sigma-Aldrich, MO, USA) was added to the diluted fibrinogen solution up to 50 mU/ml, and the mixed solution was incubated for 2 hours at 37°C. Finally, aprotinin was added to the solution

FgDP: fibrinogen degradation products

up to 1 kU/ml and mixed.

Statistical analysis

Unless otherwise indicated, all measurements are expressed as the means $\pm$ standard deviation (SD). The SPSS 15.0J statistical software package (SPSS Inc., Chicago, Illinois) was used for all statistical analyses. A Kolmogorov-Smirnov goodness of fit test was performed to determine whether the data were normally distributed. Logarithmic transformations were made for the variables if the data were not normally distributed. Comparisons were made using the one-way factorial or one-way and two-way repeated measures ANOVA. In a post-hoc analysis, the Bonferroni test was performed. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

During the observational period, the mean arterial pressures were maintained at 100~120 mmHg and hypotension was not observed in any of the

SD: standard deviation

groups. The results of the arterial blood gas analyses are presented in Table 1. Hypoxemia and lactic acidosis were not observed in the three groups. The hemoglobin level was gradually diluted by drawing blood samples and infusion of normal saline in all groups. The platelet counts and coagulation variables are presented in Table 2. The platelet counts, coagulation and fibrinolysis variables deteriorated in proportion to the doses of TF administered. In particular, the fibrinogen levels and prothrombin time in the high dose group reached the lower and upper measurement limits, respectively. The plasma alpha 2-plasmin inhibitor levels also decreased in a TF dose-dependent manner.

Increased FgDP levels were observed in the Western blot analysis. Although FgDP is composed of X, Y, D, and E fractions, the D fraction was not detected in the Western blot analysis. The differences of the FgDP in each group were statistically significant at four hours after the start of TF infusion (Figures 1 A and B). Although the amount of the E fraction of FgDP in each group was not significantly different, the high dose group showed significantly larger amounts of the X and Y fractions of FgDP than the other groups (Fig. 1 B). In the high dose group, the X and Y fractions of FgDP increased gradually, however, the E fraction was not changed (Fig. 1 C).

In the enzyme-linked immunosorbent assays, the PIC level of the high dose group was higher than those of the other groups at four hours after the start of TF infusion (Fig. 2 A). In the high dose group, the PIC levels markedly increased in proportion to the time that had passed since the beginning of the infusion (Fig. 2 B).

DISCUSSION

In the present study, TF induced the consumption of coagulation factors and alpha 2-plasmin inhibitor, fibrinolysis, and fibrinogenolysis, in proportion to the doses of TF administered. These changes can be considered to indicate DIC with the fibrinolytic phenotype. Systemic hypoperfusion-induced lactic acidosis was not observed in any of the groups during the study period. These results suggest that TF-induced fibrinogenolysis is independent of systemic hypoperfusion.

The activation of the coagulation cascade in response to TF release results in fibrin formation, triggers the activation of the fibrinolytic system, thus

leading to the generation of plasmin, which occurs in tandem with the fibrin formation (17). Consequently, the generated plasmin induces physiological fibrinolysis (17). These changes are processes associated with normal hemostasis and wound healing. However, excessive TF release induces unregulated activation of the coagulation cascade, massive fibrin formation, and results in consumption coagulopathy (10-12,16). The massive fibrin formation simultaneously induces increased plasmin generation and the consumption of alpha 2-plasmin inhibitor (12,18,19). Low levels of the alpha 2-plasmin inhibitor trigger the easy escape of plasmin from the surface of fibrin clots, and induces fibrinogenolysis. The previous studies using the TF-induced DIC model indicated that there was only consumption coagulopathy (decrease of fibrinogen level) and fibrinolysis (increase of D-dimer level) (15,16). In the present study, both marked plasmin formation detected by higher PIC levels and the consumption of alpha 2-plasmin inhibitor were observed in the group treated with the highest dose of TF, which were followed by fibrin(ogen)olysis, detected as increased levels of D-dimer, FgDP, and FDP. These changes coincided with DIC with the fibrinolytic phenotype.

Another cause of fibrinogenolysis in the high dose group may be

considered to be the increased generation of FDP. In addition to the fibrin-mediated activation of the fibrinolytic system, some of the FDP fraction could also activate fibrinolysis (17,20-22). The (DD)E complex and alpha C-domain of FDP have t-PA- and plasminogen-binding sites and preserve their competence for activating the fibrinolytic system (17,21,22). In the present study, the large amounts of FDP released into the systemic circulation may have activated the fibrinolytic system and induced fibrin(ogen)olysis in the high dose group.

The pathophysiological mechanisms underlying DIC with the fibrinolytic phenotype immediately after trauma are complicated (1). Hypoperfusion-induced hyper-fibrinolysis has been indicated as one of the important mechanisms of pathogenesis of DIC with the fibrinolytic phenotype immediately after severe trauma (1,2,4,6-8). Hypoperfusion induces the acute release of t-PA from the endothelium (23). In fact, in severe trauma patients with shock-induced systemic hypoperfusion, the acute release of t-PA was observed immediately after the occurrence of severe trauma (8,18,24). The excessively released t-PA from the endothelium causes DIC with the fibrinolytic phenotype and a tendency to haemorrhage (3,8,18,24). In observations made by thrombelastography,

excessively released t-PA resulted in early decreases of the viscoelasticity of blood clot, as was described in Kashuk's report (3).

On the contrary, Lustenberger and colleagues (25) indicated that hypoperfusion was not essential for the development of coagulopathy in patients just after severe brain trauma. Kushimoto and colleagues (26) also showed increases in the FgDP in patients with closed traumatic brain injury. In cases with simple head trauma, hypotension was not usually observed. The TF was released from injured brain tissues into the systemic circulation and could induce DIC (12,27,28). In the present study, we demonstrated that massive TF induced fibrinogenolysis, fibrinolysis and consumption coagulopathy, independent of hypoperfusion. These results from the current and previous studies suggest that increased fibrin(ogen)olysis may occur as a result of both massive TF release and shock-induced systemic hypoperfusion in severely injured trauma patients.

In the present study, the amount of the E fraction of FgDP in each group was not significantly different, although the X and Y fractions did increase in the high dose group. The molecular weight of the E fraction of FgDP is about 58kDa, which is much smaller than the molecular weights of the X and Y fractions (245kDa and 166kDa). Therefore, no significant changes in the amount of E

fraction was observed because the clearance of the E fraction was faster than that for both the X and Y fractions.

In conclusions, we demonstrated that a massive amount of TF can induce fibrinolysis and fibrinogenolysis without tissue hypoperfusion. In addition to hypoperfusion-induced t-PA release from the endothelial cells, both a decrease in the levels of alpha 2-plasmin inhibitor by consumption and an increase in the levels of plasmin may be major factors involved in the pathogenesis of hyper-fibrin(ogen)olysis after trauma. This high level of TF can further decrease the platelet counts, and lead to a prolonged prothrombin time, and consume the fibrinogen and antithrombin. These changes can therefore be called DIC with the fibrinolytic phenotype.

Conflicts of Interest and Source of Funding

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FIGURE LEGENDS

FIG. 1. The X, Y, and E fractions of fibrinogen degradation products (FgDP).

A: Representative results of the Western blot analyses showing the levels of the X, Y, and E fractions of FgDP at 4 hours after the start of TF infusion. **B:** Comparison of the levels of FgDP at 4 hours after the start of TF infusion in each group. The amounts of the X and Y fractions of FgDP in the high dose group were significantly increased compared with those of the other groups, however statistically significant differences in the E fractions of FgDP were not observed. The amount of each fraction of FgDP was demonstrated in comparison with the density of the E fraction in the positive control, which was defined as 100%. **C:** The time course of the changes in the levels of FgDP after the start of TF infusion in the high dose group. Although the E fraction of FgDP was unchanged, the levels of the X and Y fractions of FgDP increased significantly. The amount of each fraction of FgDP was demonstrated in comparison with the density of

the E fraction in the positive control, which was defined as 100%. #, $P<0.01$ vs. 0 hour; +, $P<0.01$ vs. 0 hour.

FIG. 2. The levels of plasmin-alpha 2 plasmin inhibitor complex (PIC).

A: Comparison of the levels of PIC at 4 hours after the start of TF infusion in each group. The PIC level of the high dose group was higher than those of the other groups. **B:** Changes in the levels of PIC after the start of TF infusion in the high dose group. The PIC levels increased significantly after treatment. #, $P<0.01$ vs. 0 hour; +, $P<0.01$ vs. 2 hour.

TABLE 2 Platelets and coagulation variables

	Time points			<i>P</i>
	0 h	2 h	4 h	
Platelet counts (x 10 ⁴ /μL)				
Control	70.2 ± 5.1	61.7 ± 7.3	52.4 ± 5.6	<0.001
Low dose [#]	74.2 ± 5.2	37.9 ± 7.6	18.8 ± 7.9	
High dose [#]	72.2 ± 5.3	21.0 ± 6.2	19.6 ± 7.5	
Prothrombin time (sec)				
Control	10.4 ± 0.3	10.6 ± 0.5	10.8 ± 0.2	<0.001
Low dose [*]	10.5 ± 0.6	11.2 ± 0.7	65.8 ± 59.4	
High dose ^{#, +}	10.8 ± 0.5	120.0 ± 0.0	120.0 ± 0.0	
Fibrinogen (mg/dL)				
Control	144 ± 7	134 ± 6	119 ± 12	<0.001
Low dose [*]	148 ± 10	90 ± 22	70 ± 0	
High dose [#]	138 ± 10	70 ± 0	70 ± 0	
Antithrombin (%)				
Control	74.3 ± 10.6	74.8 ± 9.6	64.0 ± 9.6	<0.001
Low dose	82.0 ± 7.6	69.0 ± 18.6	41.5 ± 12.4	
High dose ^{#, +}	70.5 ± 14.9	35.7 ± 14.1	18.0 ± 12.3	
α2-plasmin inhibitor (%)				
Control	109.6 ± 8.2	97.5 ± 7.7	83.1 ± 7.7	0.003
Low dose [#]	92.8 ± 10.1	83.3 ± 5.9	58.6 ± 8.1	
High dose [#]	106.1 ± 6.0	72.3 ± 14.2	57.2 ± 2.6	
D-dimer (ng/mL)				
Control	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	<0.001
Low dose	0.03 ± 0.00	0.05 ± 0.03	2.64 ± 6.34	
High dose ^{#, +}	0.03 ± 0.00	27.25 ± 3.57	27.67 ± 5.23	
FDP (μg/mL)				
Control	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	<0.001
Low dose [#]	0.3 ± 0.1	4.0 ± 2.6	4.7 ± 2.3	
High dose [#]	0.3 ± 0.1	23.4 ± 2.0	33.0 ± 3.6	

P values were obtained by two-way repeated measures ANOVA. In post hoc analysis, Bonferroni test was performed.

[#] *P* <0.01 vs. Control.

⁺ *P* <0.01 vs. Low dose.

^{*} *P* <0.05 vs. control.

FIGURE 1.

FIGURE 2.