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Regular Article

Plasma ADAMTS13, von Willebrand Factor (VWF) and VWF Propeptide Profiles in Patients with DIC and Related Diseases

Koji Habe [a], Hideo Wada [b,*], Naomi Ito-Habe [c], Tsuyoshi Hatada [d], Takeshi Matsumoto [e], Kohshi Ohishi [e], Kazuo Maruyama [f], Hiroshi Imai [d], Hitoshi Mizutani [a], Tsutomu Nobori [b]

[a] Department of Dermatology, Mie University Graduate School of Medicine, Mie, Tsu
[b] Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, Mie, Tsu
[c] Department of Hematology and Oncology, Mie University Graduate School of Medicine, Mie, Tsu
[d] Emergency Critical Care Center, Mie University Graduate School of Medicine, Mie, Tsu
[e] Department of Blood Transfusion, Mie University Graduate School of Medicine, Mie, Tsu
[f] Department of Anesthesia, Mie University Graduate School of Medicine, Mie, Tsu

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ABSTRACT

ADAMTS13, endothelial von Willebrand factor (VWF) and related proteins are involved in the pathogenesis of some life threatening systemic thrombotic coagulopathies. Changes of plasma ADAMTS13 activity in thrombotic thrombocytopenic purpura (TTP) is well known but is also involved in septic disseminated intravascular coagulation (DIC). Here we investigated the ADAMTS13 activity, VWF and VWF propeptide (VWFpp) antigens in 69 patients with DIC, 143 with non-DIC, 21 with thrombotic thrombocytopenic purpura (TTP) and 23 with atypical hemolytic uremic syndrome (aHUS) for diagnosis of DIC.

The plasma ADAMTS13 activity was significantly low in patients with DIC, and the plasma levels of VWF and VWFpp antigens, were the highest in these patients, but there were no significant differences in the plasma VWFpp levels between the patients with DIC and those with aHUS. The difference in the plasma ADAMTS13 activity, the VWF and VWFpp antigens between DIC and non-DIC cases was significant in those with infectious and malignant diseases, but the difference in the VWFpp/ VWF ratio were significant only in subjects with infectious diseases. As an indicator for prognosis, the plasma levels of VWFpp were significantly higher in non-survivors than in survivors. Then, VWFpp/ VWF ratio and VWFpp/ADAMATS13 ratio will be potent informative indicators in DIC.

These findings suggest that ADAMTS13/VWF profiles may have important roles in the pathogenesis of DIC, and that ADAMTS13 and VWFpp are useful indicators for the diagnosis and prognosis of DIC.

Introduction

Figs. 2 and 4

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13) is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF) [1–5]. A severe deficiency in the ADAMTS13 activity is caused by either a mutation of the ADAMTS13 gene [2, 6] or by the presence of inhibitory antibodies against ADAMTS13 [7]. Unusually large VWF multimers (UL-VWFMs) produced and released from the injured vascular endothelial cells to the plasma of patients with familial and nonfamilial thrombotic thrombocytopenic purpura (TTP) [8, 9].

The pre-pro VWF, which is synthesized in endothelial cells and megakaryocytes, undergoes intracellular modifications including signal peptide cleavage, C-terminal dimerization, glycosylation, sulfation, and N-terminal multimerization [10]. Then proteolysis occurs in the trans-Golgi where the VWF propeptide (VWFpp) is cleaved but remains stored together with mature VWF in alpha-granules (megakaryocytes) and Weibel-Palade bodies (endothelial cells). After the secretion of VWFpp and VWF into plasma from endothelial cells in response to several physiological or pathological stimuli, VWFpp dissociates from VWF [11, 12]. An elevated plasma level of VWFpp has been reported in patients with thrombotic microangiopathy (TMA) [13]. Disseminated intravascular coagulation (DIC) is a life-threatening disease that is often associated with severe organ failure and a bleeding tendency [14]. DIC is diagnosed based on the clinically laboratory coagulation tests but are not sensitive for the early phase of DIC. Thus, a new marker is required for the diagnosis of DIC [14]. Decreased ADAMTS13 levels were previously reported in the patients with septic DIC [15, 16].

In this study, we measured the ADAMTS13 activity, and the VWFpp and VWF antigens in the plasma from 69 patients with DIC, 143 with non-DIC, 21 with TTP and 23 with atypical hemolytic uremic syndrome (aHUS) to evaluate usefulness in diagnosing DIC.

* Corresponding author at: Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, 2–174 Edobashi, Tsu, Mie 514–8507, Japan. Tel.: +81 59 232 1111; fax: +81 59 231 5204.
E-mail address: wadahide@clin.medic.mie-u.ac.jp (H. Wada).

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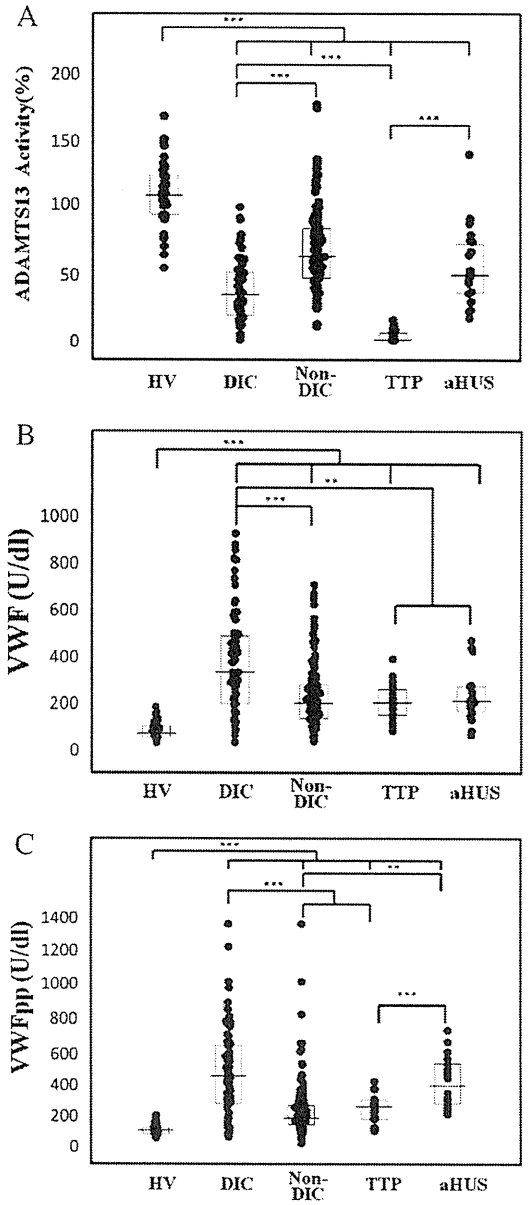

Fig. 1. The plasma levels of ADAMTS13 activities, the VWF and VWFpp antigens in healthy volunteers, DIC, non-DIC, TTP and aHUS patients. **A.** The plasma ADAMTS13 activities. ***; $p<0.001$, **; $p<0.01$, *; $p<0.05$. **B.** The plasma levels of the VWF antigen. ***; $p<0.001$, **; $p<0.01$. **C.** The plasma levels of the VWFpp antigen. ***; $p<0.001$, **; $p<0.01$. HV; healthy volunteers, DIC; disseminated intravascular coagulation, TTP; thrombotic thrombocytopenic purpura, aHUS; atypical hemolytic uremic syndrome.

Materials And Methods

The ADAMTS13 activity, VWF antigen and VWF propeptide (VWFpp) were measured in 50 healthy volunteers, 69 patients with DIC, 143 without DIC (non-DIC), 21 with TTP and 23 with aHUS. The DIC patients and non-DIC patients were continuously selected from the patients with a platelet count<100,000/μl from January 1, 2010 until December 31, 2010 at the Mie University Hospital. The patients were classified into three groups: patients with infectious diseases (25 with DIC and 30 with non-DIC), with malignant diseases (30 with DIC and 57 with non-DIC), and with other diseases (14 with DIC and 56 with non-DIC). In other diseases, there were 26 patients with an aneurysm (5 with DIC), 16 patients with trauma (4 with DIC), 13 patients with heart diseases (3 with DIC), 10 patients with

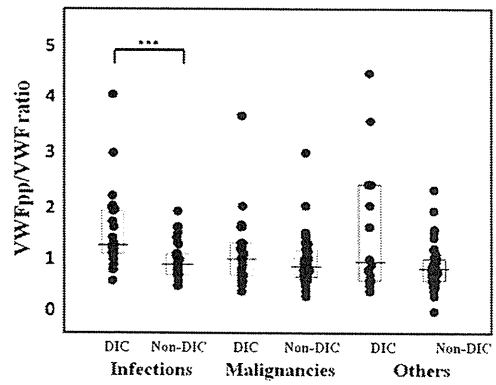

Fig. 2. The VWFpp/VWF ratio in healthy volunteers, DIC, non-DIC, TTP and aHUS patients. **; $p<0.001$.

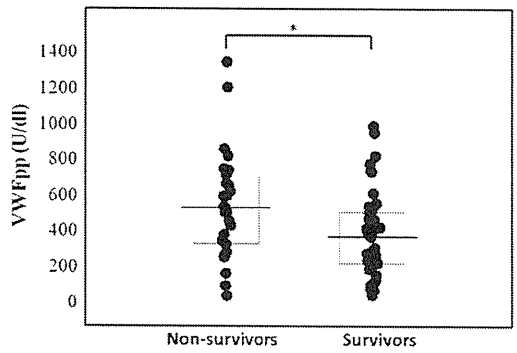

Fig. 3. The plasma levels of VWFpp in non-survivors and survivors. *; $p<0.05$.

digestive diseases (2 with DIC) and 5 patients with autoimmune diseases (no with DIC). DIC was diagnosed by the overt-DIC diagnostic criteria established by the International Society of Thrombosis Haemostasis (ISTH)[17]. TMA, which results in thrombocytopenia and hemolytic anemia due to the microangiopathy, was identified by based on the laboratory data and clinical symptoms such as neurological dysfunction, renal failure, or fever [18]. TTP was diagnosed when a patient had TMA and less than 10% of the normal ADAMTS 13 activity, and aHUS was diagnosed when a patient had TMA and reduced, but more than 10% of the normal level, of ADAMTS13 activity. No patients with TTP and 5 patients with HUS were diagnosed to have overt-DIC. The 69 patients with DIC were treated according to the

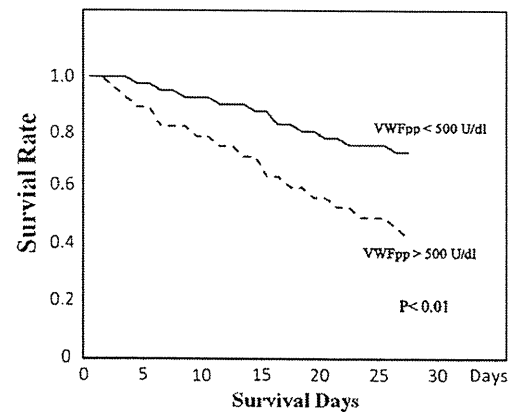

Fig. 4. Kaplan-Meier survival analysis of DIC patients with more than 500 U/dl of VWFpp and those with less than 500 U/dl of VWFpp.

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Table 1
Characteristics of the Study Subjects.

	N	Age	Sex (F : M)	ADAMTS13 (%)	VWF (U/dl)	VWFpp (U/dl)	VWFpp/VWF Ratio	VWFpp/ADAMTS13 Ratio (U/%)
Healthy volunteers	50	31 (19.0 – 51.0)	31 : 19	110.0 (95.0 – 125.0)	69.5 (55.0 – 102.0)	85.0 (62.0– 102.0)	1.12 (0.93 – 1.27)	0.77 (0.60 –0.97)
DIC	69	65 (55.0 – 73.0)	24 : 45	35.0 (19.5 – 51.6)	338 (200 – 496)	421 (251 – 608)	1.10 (0.78 – 1.62)	19.9 (5.4 –28.5)
Non-DIC	143	63 (46.0 – 71.8)	59 : 84	63.7 (47.5 – 85.0)	203 (136 – 286)	162 (121 – 239)	0.85 (0.64 – 1.05)	2.06 (1.25 – 5.47)
TTP	21	65 (40.5 – 75.5)	11 : 10	UD (UD – 6.3)	206 (153 – 265)	233 (156 – 273)	1.07 (0.86 – 1.20)	
aHUS	23	40 (28.5 – 54.0)	17 : 6	50.0 (36.6 – 73.1)	214 (170 – 277)	361 (249 – 497)	1.13 (1.00 – 1.69)	4.9 (3.0-9,5)

The data are shown as the medians (25 – 75 percentile), UD; undetectable.

Table 2
The Differences in the Plasma Levels of ADAMTS13, VWF, VWFpp and the VWFpp/VWF Ratio Between DIC and non-DIC Cases.

		ADAMTS13 (%)	VWF (U/dl)	VWFpp (U/dl)	VWFpp/VWF Ratio
Infectious diseseas	DIC	30.0** (15.9 – 49.4)	382* (286 – 496)	545*** (363 – 730)	1.26*** (1.10 – 1.91)
	Non-DIC	50.7** (36.3 – 68.8)	294* (202 – 387)	241*** (169 – 325)	0.90*** (0.70 – 1.10)
Malignancies	DIC	31.9*** (22.5 – 51.3)	384*** (198 – 598)	398*** (193 – 512)	1.00 (0.70 – 1.30)
	Non-DIC	63.5*** (51.3 – 76.9)	170*** (122 – 279)	143*** (117 – 239)	0.87 (0.67 – 1.16)
Other diseases	DIC	48.8** (28.8 – 61.3)	180 (121 – 407)	269* (119 – 463)	0.95 (0.60 – 2.40)
	Non-DIC	80.7** (55.7 – 95.0)	196 (140 – 255)	144* (109 – 199)	0.83 (0.60 – 1.01)

***; p<0.001, **; p<0.01, *; p<0.05 for the significance between DIC and non-DIC cases.

Expert Consensus for the Treatment of DIC [19]. Twenty-seven patients died within 28 days after the diagnosis of DIC (non-survivor) but 42 patients survived after the 28 days (survivor). For the non-survivor group, the median (25-75th) survival was 16 days (7.5-21.5 days). The study protocol was approved by the Human Ethics Review Committee of Mie University School of Medicine and a signed consent form was obtained from each subject.

Human plasma was obtained from whole blood that was treated with a 1/10 volume of 3.8% sodium citrate as an anti-coagulant by centrifugation at 3,000 x g at 4 °C for 15 min. The plasma was stored at −80 °C until analysis.

The ADAMTS13 activity level was measured using a FRETS-VWF73 peptide, which was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) according to the method reported by Kokame *et al.*[20]. The plasma levels of VWF and VWFpp were measured with a VWF & Propeptide assay kit (GTi DIAGNOSTiCs, Waukesha, USA) [13]. The hemoglobin (Hb) levels and platelet counts were measured by automated the fully automated hematology analyzer XE-2100 (Sysmex, Kobe, Japan).

Statistical analysis

The data are expressed as the medians (25-75th percentile). The differences between the groups were examined for statistical significance using the Mann–Whitney *U* test. A *P* value<0.05 denoted the presence of a statistically significant difference.

Results

The plasma ADAMTS13 activities were significantly decreased in any of patients with DIC, non-DIC, TTP and aHUS compared with those in healthy volunteers (Fig. 1-A and Table 1). Although the plasma ADAMTS13 activity in patients with DIC was significantly lower than in those with non-DIC, however, it is still significantly higher than that of TTP. The plasma levels of VWF and VWFpp antigens were significantly elevated in patients with DIC, non-DIC, TTP and aHUS compared with that of healthy volunteers (Fig. 1-B and 1-C, and Table 1). The plasma VWF antigen level in patients with DIC was higher than other groups. The plasma VWFpp antigen level in DIC was significantly higher than in those with non-DIC and TTP. Although there were no significant differences between patients with DIC and those with aHUS, the VWFpp/ADAMTS13 ratio was significantly higher in DIC (19.9: 5.4 –28.5) than in aHUS (4.9: 3.0 – 9.5, p<0.01).

The difference in the plasma ADAMTS13 activity and the level of VWFpp antigen between DIC and non-DIC cases was significant for those with infectious diseases (p<0.01 and p<0.001, respectively), malignant diseases (p<0.001, respectively) and other diseases (p<0.01 and p<0.05, respectively, Table 2). The difference in the plasma VWF antigen level between DIC and non-DIC cases was limited in those with infectious diseases (p<0.05) and malignant diseases (p<0.001). Therefore, a significant elevation of the VWFpp/VWF ratio was observed between DIC and non-DIC groups with infectious diseases.

The plasma ADAMTS13 activity was negatively correlated with the DIC score. The plasma levels of VWF, VWFpp and the VWFpp/VWF ratio were also correlated with the DIC score (Table 3). The plasma ADAMTS13 activities were lower in non-survivors than in survivors without significance. However, the plasma levels of VWFpp were significantly higher in non-survivors than in survivors (p<0.05) (Table 4 and Fig. 3). Therefore, the ratio of VWFpp/ADAMTS13 was significantly higher in non-survivors (26.4: 9.9 – 43.2) than in survivors (8.6: 5.3 – 16.5, p<0.01). In a Kaplan- Meier survival analysis, the survival rate was significantly higher in the DIC patients with less than 500 U/dl of VWFpp than in those with more than 500 U/dl of VWFpp (p<0.01). There were no significant differences in the VWF antigen level and the DIC score between non-survivors and survivors.

Discussion

In the present study, the plasma ADAMTS13 activities in patients with DIC were significantly lower than those with non-DIC, but were significantly higher than in those with TTP. Decrease of ADAMTS13 activity was reported in patients with sepsis-induced DIC [15] and severe DIC [16]. The number of DIC patients with infections was higher in this study (n=25) than that described in Dr Hyun's report (n=5)[16] and the cut-off value of fibrin related markers in the diagnostic criteria for overt-DIC was lower in their study (D-dmier: 2 points >0.4 μg/ml and 3 points>4 μg/ml) than in

Table 3
The Relationship Between the Plasma Levels of ADAMTS13 Activity, VWF, VWFpp and the VWFpp/VWR Ratio with the DIC Score.

	r	95%CI	
ADAMTS13	- 0.309	- 0.426~— 0.182	P<0.001
VWF	0.308	0.181~0.425	P<0.001
VWFpp	0.447	0.333~0.549	P<0.001
VWFpp/VWF ratio	0.337	0.212~0.452	P<0.001

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Table 4
Plasma Levels of ADAMTS13, VWF, VWFpp, the VWFpp/VWF Ratio and the DIC Score in Survivors and Non-survivors.

	ADAMTS13 (%)	VWF (U/dl)	VWFpp (U/dl)	VWFpp/VWF Ratio	VWFpp/ADAMTS13 Ratio(U/%)	DIC score
Non-survivors (N=27)	25.0 (15.3 – 50.1)	338 (214 – 487)	538* (336 – 709)	1.30 (0.81 – 1.99)	26.4 (9.9 – 43.2)	5 (5 – 6)
Survivors (N=42)	37.6 (26.0 – 53.6)	351 (168 – 493)	377* (225 – 512)	1.05 (0.70 – 1.30)	8.6** (5.3 – 16.5)	5 (5 – 5)

*; $p<0.05$ for significance between Non-survivor and Survivor.

this study (FDP: 2 points > 10 μg/ml and 3 points > 40 μg/ml), thus suggesting that the number of DIC patients with infections and the severity (mortality) of DIC were both higher in this study than in that previous report. UL-VWFMs freshly discharged from endothelial cells is a substrate for ADAMTS13, and were observed in those with sepsis-induced DIC [15]. In TTP, ADAMTS13 is the primary target of the autoimmunity, and loss of its function results in systemic manifestations of TTP. Impaired ADAMTS13 activity related to renal failure [15], and correlated with a poor outcome [16] in DIC. A few patients with aHUS were found to satisfy the diagnostic criteria for ISTH overt DIC. Thus, DIC and TTP may be sharing a common pathogenesis, especially in platelet hyper aggregation.

Significant reductions in the ADAMTS13 activity and increases in the plasma level of the VWF antigen were reported in patients with TMA due to liver transplantation [21]. ADAMTS13 is mainly produced in liver and discharged into blood. Temporally loss of liver function at liver transplantation decreased ADAMTS13 production and the decreased plasma ADAMTS13 levels induced a TTP like condition with the surgical stress. DIC, TTP, and TMA due to transplantation may have common pathological condition based on loss of ADAMTS13 activities. Different from TTP, mechanism of plasma ADAMTS13 decrease in DIC is not clearly declared. Degradation by neutrophil elastase has been suggested for the pathogenesis of decrease in ADAMTS13 associated with sepsis [15].

VWF is produced and released from vascular endothelial cells, and is considered as a marker of vascular endothelial cell injury [14]. VWF is a substrate for platelet aggregation and is reduced in cases of TTP. The plasma levels of the VWFpp antigen were significantly higher in patients with DIC than in those with non-DIC and those with TTP. VWFpp are discharged from impaired endothelial cells as well as VWF. However, VWFpp is not consumed by platelet aggregation, and the plasma VWFpp level more directly reflects vascular endothelial cell injury than that of VWF. aHUS is a disease with severe endothelial cell damage. The plasma VWFpp level in the present study is significantly elevated as reported previously [13]. It is difficult to differentiate DIC from aHUS simply based on the VWFpp levels. However, VWFpp/ADAMATS13 ratio is a potent indicator for differentiation between DIC and aHUS.

Between DIC and non-DIC cases, significant difference is present in the plasma ADAMTS13 activity, VWFpp antigen level and VWFpp/VWF ratio. DIC develops in both of malignancy and infections diseases. However, increase of VWFpp/VWF ratio is obvious in patients with infectious diseases but not with malignancy. Consumption of VWF by severe sepsis may represent this difference, and this ratio is a potent marker for DIC of infection. Therefore, the ADAMTS13/VWF system may play an important role in the onset of DIC in patients with infectious diseases. A prognostic index in DIC is important information for clinicians. Present study indicated significant increase of the plasma levels of VWFpp in non-survivors than that of survivors. ADAMTS13 activities in non-survivors is also lower that of survivors without significance. Decreased ADAMTS13 and increased VWFpp levels may reflect a poor outcome with severe microangiopathy and organ failure. In the present study, the increased VWFpp/ADAMTS13 ratio clearly differentiated the non-survivors from the survivors, and it will be a potent useful marker for prediction of prognosis in patients with DIC.

The marked decrease in the ADAMTS13 activity is one of the main causes of TTP, a life-threatening syndrome characterized by thrombocytopenia and microangiopathic hemolytic anemia, and is often associated with neurological dysfunction, renal failure, and fever [22] due to marked platelet activation. Elevated VWFpp was also reported to be related to a poor outcome in TMA [13]. Therefore, the septic DIC and TMA may have a common pathogenesis: platelets hyper-activation with vascular endothelial cell injuries, and poor outcome.

In conclusion, markedly increased levels of VWF and decreased ADAMTS13 activity might play an important role in the pathogenesis of DIC, and increased plasma level of VWFpp and decreased ADAMTS13 activity may be related to a poor outcome in DIC patients.

Conflict of interests statement

All authors have no conflict interest.

Acknowledgments

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ORIGINAL ARTICLE

Increased fibrinolysis increases bleeding in orthopedic patients receiving prophylactic fondaparinux

Kakunoshin Yoshida · Hideo Wada · Masahiro Hasegawa · Hiroki Wakabayashi · Takeshi Matsumoto · Yuji Shimokariya · Katsura Noma · Norikazu Yamada · Atsumasa Uchida · Tsutomu Nobori · Akihiro Sudo

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Abstract We evaluated hemostatic markers in patients who underwent major orthopedic surgery, including total hip and total knee arthroplasty, and were treated for the prophylaxis of deep vein thrombosis (DVT) with or without fondaparinux (anti-Xa group, $n = 98$ and without anti-Xa group, $n = 20$). The frequency of DVT was significantly higher in the without anti-Xa group than in the anti-Xa group, but the reduction of hemoglobin and fibrinolytic marker levels was significantly lower in the without anti-Xa group than in the anti-Xa group. Eighteen patients in the anti-Xa group showed a reduction in hemoglobin of more than 2 g/dl, and those individuals were considered to be the increased bleeding (IB) group. The concentration of fibrinolytic markers in the anti-Xa group was significantly higher in the IB group than in the non-IB group. There were also no significant differences in the levels of anti-Xa activity, plasminogen activator inhibitor-I, soluble fibrin and antithrombin between the IB and non-IB groups. In conclusion, elevated fibrinolysis induced by increased bleeding may lead to further increases in bleeding in patients receiving thromboprophylaxis with fondaparinux following major orthopedic surgery.

Keywords Deep vein thrombosis (DVT) · Fibrinolysis · Fondaparinux · Orthopedic surgery · Bleeding

K. Yoshida · M. Hasegawa · H. Wakabayashi · A. Sudo
Department of Orthopaedic Surgery, Mie University Graduate School of Medicine, Tsu, Japan

H. Wada (✉) · T. Nobori
Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie-ken 514-8507, Japan
e-mail: wadahide@clin.medic.mie-u.ac.jp

T. Matsumoto
Department of Blood Transfusion, Mie University Graduate School of Medicine, Tsu, Japan

Y. Shimokariya · K. Noma
Department of Central Laboratory, Mie University Graduate School of Medicine, Tsu, Japan

N. Yamada · A. Uchida
Department of Cardiology and Nephrology, Mie University Graduate School of Medicine, Tsu, Japan

Introduction

Fibrin-related markers such as D-dimer, fibrin and fibrinogen degradation products (FDP) and soluble fibrin (SF) are useful for the diagnosis of thrombosis, and are elevated in deep vein thrombosis (DVT)/pulmonary embolism (PE) and disseminated intravascular coagulation (DIC) [1–5]. PE is a common, frequently undiagnosed and potentially fatal cause of several common symptoms: dyspnea and chest pain [6–8]. Preventing the development of DVT is clinically important, because PE is often a fatal disease caused by DVT. Orthopedic surgery is associated with a very high rate of postoperative venous thromboembolism (VTE) [9, 10]. The incidence of venographically proven VTE ranges from 42 to 57% after total hip arthroplasty (THA) surgery in the absence of thromboprophylaxis, and 41 to 85% after total knee arthroplasty (TKA) surgery [11]. Multiple studies [12–14] have established the usefulness of low molecular weight heparin (LMWH) for VTE prophylaxis in orthopedic surgery patients.

Fondaparinux is the first selective factor Xa inhibitor approved for use in thromboprophylaxis after orthopedic surgery [15–17], and studies comparing fondaparinux to LMWH showed very useful thromboprophylaxis in

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patients after orthopedic surgery [16, 17]. There are a few cases with massive bleeding in patients administered fondaparinux. Therefore, fondaparinux is frequently administered at a dose of 1.5 mg instead of 2.5 mg in Japan to avoid serious bleeding. Anti-Xa activity has been measured as UFH or LMWH activity to monitor the anticoagulant activity [18, 19].

This study evaluated the hemostatic markers in patients who underwent major orthopedic surgery and were treated for the prophylaxis of DVT with or without fondaparinux ($n = 98$ and $n = 20$) to examine the fibrinolytic activity in patients with major bleeding.

Materials and methods

From 1 January 2007 to 30 March 2007, 20 patients (median age 67.5 years of age, 25–75% range, 63.5–75.0 years of age; 16 females and 4 males) underwent orthopedic surgery (14 THA and 6 TKA) and were treated with only intermittent pneumatic compression for prophylaxis of DVT (the without anti-Xa group). Ninety-eight orthopedic patients treated with 1.5 mg of fondaparinux (GlaxoSmithKine, Tokyo, Japan) and intermittent pneumatic compression for prophylaxis of DVT from 1 February 2010 to 31 December 2010 were enrolled in this study (the with anti-Xa group). These patients received 1.5 mg of Fondaparinux by hypodermic injection once a day for 14 days beginning 24 h after extubation of lumbar anesthesia. There were no significant differences in the age, sex and type of operation between the without and with anti-Xa groups (Table 1). A reduction of more than 2 g/dl of hemoglobin from day 1 to 14 was observed in 18 patients (increased bleeding group; IB group; Table 2). No patients with IB received blood transfusion and none had associated DIC. There was no significant difference in the age, weight, height, body mass index (BMI), body surface area, estimated glomerular filtration rate (eGFR), hemoglobin and antithrombin (AT) before surgery between patients with and without IB. Only the creatinine concentration and number of males were higher in patients with IB than in those without IB. Anti-Xa activity, fibrin and FDP, D-dimer, soluble fibrin (SF) and AT activity were measured in 73 patients after THA and 23 patients after TKA before and on days 1, 4, 8 and 15 of the administration of fondaparinux. The study protocol was approved by the Human Ethics Review Committee of the Mie University School of Medicine and a signed consent form was obtained from each subject. This study was faithfully carried out in accordance with the Declaration of Helsinki.

The anti-Xa activity was monitored 3 h after injection of fondaparinux. The anti-FXa activity of fondaparinux was

Table 1 Subjects with and without anti-Xa treatment

	Without anti-Xa	With anti-Xa
Age (years)	67.5 (63.5–75.0)	68.0 (61.0–75.0)
Female:male	16:4	75:23
THA:TKA	14:6	73:25
DVT	10/20 (50%)**	16/98 (14.3%)**
Reduction of hemoglobin (g/dl)	0.55 (0.00–1.10)***	2.20 (1.60–3.28)***
Weight (kg)	56.8 (49.7–65.2)	57.1 (50.1–66.9)
Creatinine (mg/dl)	0.68 (0.54–0.79)	0.67 (0.56–0.80)
Underlying diseases	Osteoarthritis[a]	Osteoarthritis[a]

THA total hip arthroplasty, *TKA* total knee arthroplasty

[a] Patients with rheumatoid arthritis were excluded from this study

** $p < 0.01$, *** $p < 0.001$

Table 2 Subjects treated with anti-Xa therapy

	Reduction by >2.0 g/dl of Hb	Reduction by <2.0 g/dl of Hb
Age (years)	71.5 (63.0–79.0)	68.0 (61.0–73.5)
Weight (kg)	53.9 (48.2–69.3)	57.4 (50.6–66.9)
High (cm)	155.9 (146.1–164.0)	151.5 (147.7–158.0)
BMI (kg/m^2)	22.5 (20.4–25.2)	24.5 (22.6–27.3)
Body surface area (cm^2)	1.50 (1.41–1.71)	1.53 (1.44–1.68)
Creatinine (mg/dl)	0.82 (0.60–1.03)*	0.64 (0.56–0.77)*
eGFR	68.9 (51.1–83.0)	76.5 (59.6–86.5)
Hb (g/dl)	12.1 (10.9–13.4)	12.2 (11.4–12.8)
AT (%)	79.5 (68.5–84.1)	81.6 (73.4–89.8)
Female:male	10:8*	65:15*
THA:TKA	14:4	59:21

THA total hip arthroplasty, *TKA* total knee arthroplasty

* $p < 0.05$

measured using Testzym® Heparin S (Sekisui Medical Co. Ltd., Tokyo, Japan) and a Coagrex® 800 (Sysmex Co. Ltd., Kobe, Japan).Testzym® Heparin S contains bovine FXa (71 nkat/vial), AT (10 IU/vial), chromogenic substrate (S-2222: Benz-Ile-Glu-Gly-Arg-pNA HCl 25 mg), pooled lyophilized normal plasma and buffer (pH 8.4) [18, 19]. A standard curve was constructed for lyophilized normal plasma using various concentrations of fondaparinux.

The reagents and objects were loaded into the Coagrex 800, and the anti-FXa activity of fondaparinux was measured automatically. A 135-μl aliquot of FXa was added to 8 μl of plasma (with diluent solution added in advance) and 75 μl of substrate was added. The released p-NA was measured photometrically at 405 nm. The anti-FXa activity of fondaparinux was then calculated using the standard curve.

Plasma levels of FDP, D-dimer, SF, plasmin plasmin inhibitor complex (PPIC) and plasminogen activator inhibitor-I (PAI-I) were measured by the latex agglutination method using Nanopia FDP, Nanopia D-dimer, Nanopia SF (Sekisui Medical), LPIAACE PPIII (Mitsubishi Chemical Medience, Tokyo, Japan) and LPIA-tPAI test (Mitsubishi Chemical Medience), respectively [20]. The plasma levels of AT were measured using a Testzym S AT III kit (Sekisui Medical). Diagnosis of DVT was assessed by whole-leg compression ultrasound examination using a standardized ultrasound criterion of venous noncompressibility before the operation, as well as on days 4 and 14 [21].

Statistical analysis

The data are expressed as the medians (25–75 percentile). Any differences between the groups were examined using the Mann–Whitney U test. The correlations between the reduction of hemoglobin and hemostatic markers were examined using the Spearman's rank correlation coefficient. A p value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo).

Results

DVT was observed in 10 patients (50%) including 3 patients with proximal DVT in the without anti-Xa group and 16 patients (14.3%) including one patient with proximal DVT in the with Xa group, indicating that the frequency of DVT was significantly higher in the without anti-Xa group than in the with anti-Xa group ($p < 0.01$). Symptomatic PE was not observed in either group. As these DVT were not observed before the operations, they were therefore considered to be fresh DVT. The reduction of hemoglobin from day 1 to 14 was significantly higher in the with anti-Xa group than in the without anti-Xa group (Table 1, $p < 0.001$).

The plasma levels of FDP were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 4 (12.0 μg/ml: 9.6–14.1 μg/ml vs. 10.0 μg/ml: 9.1–11.0 μg/ml, $p < 0.05$), day 8 (14.9 μg/ml: 11.2–19.7 μg/ml vs. 10.8 μg/ml: 8.4–13.6 μg/ml, $p < 0.001$) and day 15 (15.1 μg/ml: 11.0–20.5 μg/ml vs. 10.2 μg/ml: 6.9–12.5 μg/ml, $p < 0.001$, Fig. 1a). The plasma levels of D-dimer were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 8 (8.2 μg/ml: 5.6–10.4 μg/ml vs. 6.3 μg/ml: 4.9–7.5 μg/ml, $p < 0.05$) and day 15 (7.7 μg/ml: 5.6–11.7 μg/ml vs. 5.7 μg/ml: 4.0–12.5 μg/ml, $p < 0.01$, Fig. 1b). The plasma levels of PPIC were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 4 (2.68 μg/ml: 2.23–3.16 μg/ml vs. 1.13 μg/ml: 0.96–1.25 μg/ml, $p < 0.001$) and day 8 (1.61 μg/ml: 1.50–2.23 μg/ml vs. 1.13 μg/ml: 0.92–1.24 μg/ml, $p < 0.01$, Fig. 1c). The plasma levels of SF were significantly higher in the with anti-Xa group than in the without anti-Xa group on day 4 (11.8 μg/ml: 8.2–17.1 μg/ml vs. 7.2 μg/ml: 5.6–8.7 μg/ml, $p < 0.001$), day 8 (7.0 μg/ml: 4.6–11.2 μg/ml vs. 4.6 μg/ml: 3.5–6.3 μg/ml, $p < 0.05$) and day 15 (4.6 μg/ml: 3.0–8.3 μg/ml vs. 3.3 μg/ml: 2.5–5.0 μg/ml, $p < 0.05$, Fig. 1d).

The concentration of hemoglobin in the anti-Xa group was significantly lower in the IB group than in the non-IB group on day 4 (8.6 g/dl: 7.9–9.0 g/dl vs. 10.3 g/dl: 9.2–11.4 g/dl, $p < 0.001$) and day 8 (9.0 g/dl: 8.4–9.7 g/dl vs. 10.3 g/dl: 9.4–11.2 g/dl, $p < 0.001$). There was no significant difference in the anti-Xa activity between the IB and non-IB groups before and on days 1, 4 and 8 of administration (Table 3; Fig. 2). The concentration of hemoglobin was significantly lower in the IB group than in the non-IB group on days 4 and 8 ($p < 0.001$).

The concentration of FDP in the anti-Xa group was significantly higher in the IB group than in the non-IB group before (31.3 μg/ml: 14.6–46.7 μg/ml vs. 15.8 μg/ml: 9.7–27.3 μg/ml, $p < 0.01$) and on day 1 (17.2 μg/ml: 11.2–27.1 μg/ml vs. 12.3 μg/ml: 9.0–17.6 μg/ml, $p < 0.05$) and day 8 (21.1 μg/ml: 17.4–25.0 μg/ml vs. 14.5 μg/ml: 11.2–18.9 μg/ml, $p < 0.05$; Fig. 3a). The concentration of D-dimer in the anti-Xa group was significantly higher in the IB group than in the non-IB group on day 0 (17.3 μg/ml: 6.5–27.4 μg/ml vs. 7.8 μg/ml: 4.6–14.4 μg/ml, $p < 0.01$), day 1 (8.2 μg/ml: 6.7–8.2 μg/ml vs. 5.8 μg/ml: 3.8–9.1 μg/ml, $p < 0.05$) and day 8 (11.4 μg/ml: 9.5–13.7 μg/ml vs. 8.1 μg/ml: 5.5–10.3 μg/ml, $p < 0.05$; Fig. 3b). No significant difference was observed regarding PAI-I, SF and AT between the IB and the non-IB groups before and on days 1, 4 and 8 of administration (Table 4). The concentration of PPIC was significantly higher in the IB group than in the non-IB

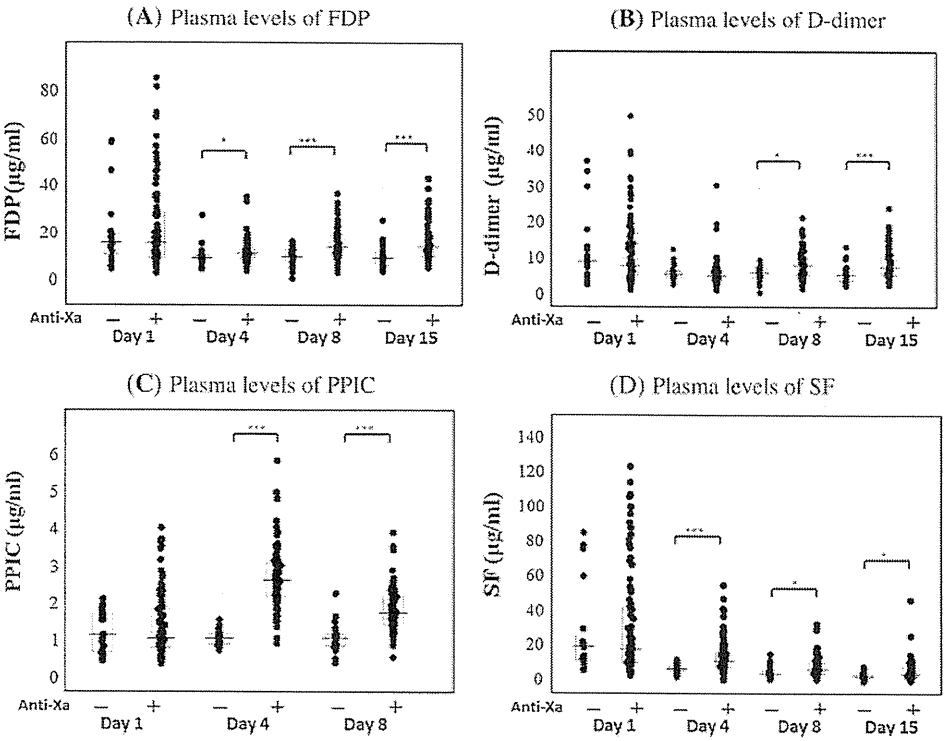


Fig. 1 The plasma levels of FDP, D-dimer, PPIC and SF in the orthopedic patients treated with and without fondaparinux. The plasma levels of **a** FDP, **b** D-dimer, **c** PPIC, and **d** SF *FDP* fibrinogen and fibrin degradation products, *PPIC* plasmin plasmin inhibitor complex, *SF* soluble fibrin; − without fondaparinux, + with fondaparinux. *$p < 0.05$, ***$p < 0.001$

Table 3 The anti-Xa activity between the patients with and without a reduction in Hb by >2.0 g/dl

Fondaparinux (mg/l)	Reduction of Hb >2.0 g/dl	Reduction of Hb <2.0 g/dl
Before	0.02 (0.00–0.06)	0.03 (0.00–0.07)
Day 1	0.31 (0.27–0.36)	0.30 (0.25–0.38)
Day 4	0.41 (0.35–0.51)	0.39 (0.34–0.50)
Day 8	0.52 (0.44–0.60)	0.46 (0.38–0.54)
Day 15	0.24 (0.10–0.54)	0.22 (0.17–0.26)

group on day 0 (1.5 μg/ml: 1.2–2.5 μg/ml vs. 1.1 μg/ml: 0.8–1.6 μg/ml, $p < 0.05$). The concentration of PPIC was highest at day 4, but there was no significant difference between the IB and non-IB groups (2.7 μg/ml: 2.3–3.1 μg/ml vs. 2.7 μg/ml: 2.2–3.2 μg/ml; Fig. 3c). The correlation with reduction of hemoglobin was more significant in PPIC, FDP and D-dimer levels than in SF levels (Table 5).

Discussion

The frequency of DVT was significantly higher in the without anti-Xa group than in the anti-Xa group, but the reduction of hemoglobin was significantly lower in the without anti-Xa group than in the anti-Xa group, indicating that fondaparinux is useful for the prophylaxis of DVT, but may increase bleeding. In the anti-Xa group, the

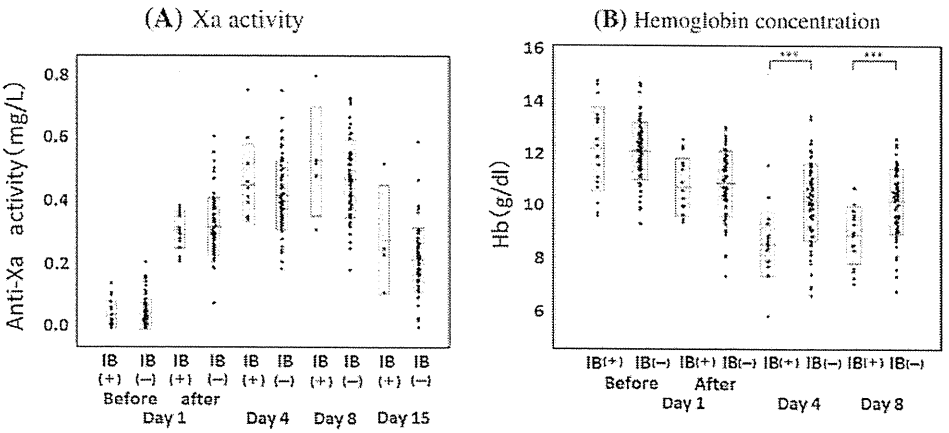
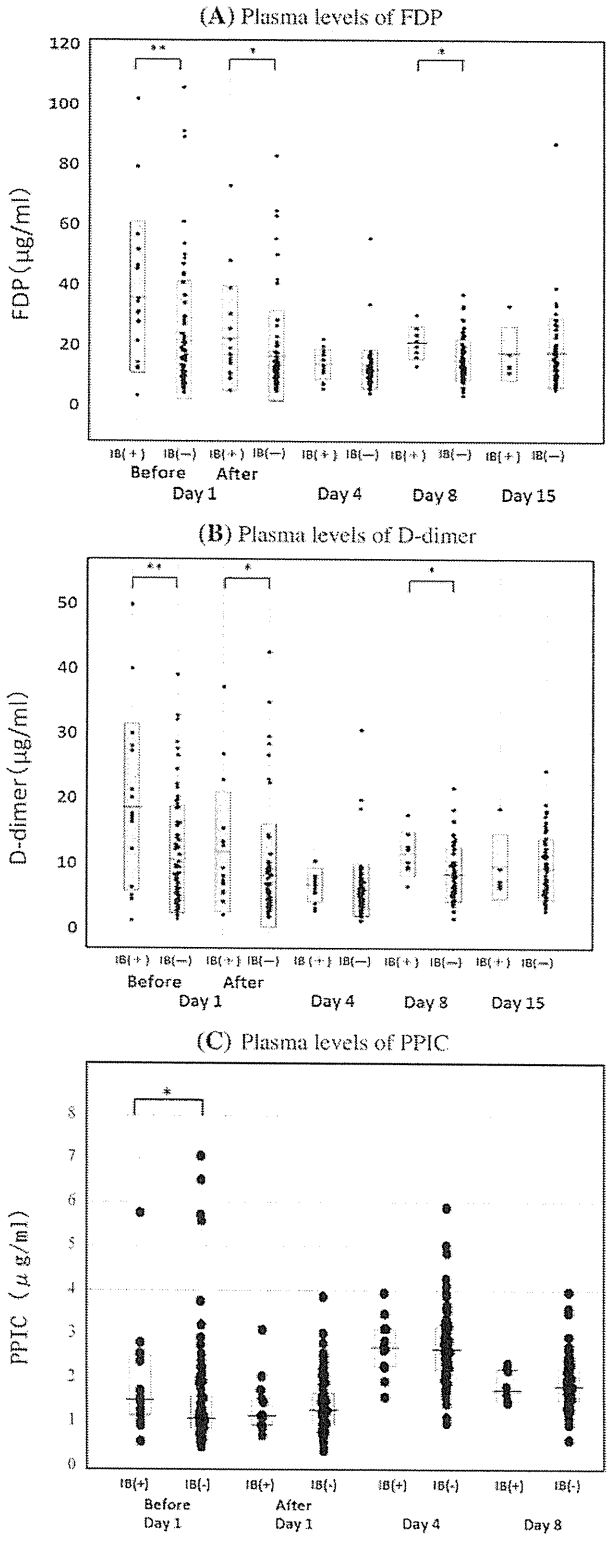


Fig. 2 Anti-Xa activities and hemoglobin concentrations in orthopedic patients treated with fondaparinux. **a** The anti-Xa activity and **b** hemoglobin concentration. *IB* increased bleeding: a reduction of more than 2 g/dl of hemoglobin, *Before* before the administration of fondaparinux, *After* after the administration of fondaparinux. ***$p < 0.001$



◀ **Fig. 3** The concentration of FDP, D-dimer and PPIC in orthopedic patients treated with fondaparinux. The plasma levels of **a** FDP, **b** D-dimer, **c** PPIC *FDP* fibrinogen and fibrin degradation products, *PPIC* plasmin plasmin inhibitor complex, *IB* increased bleeding: a reduction of hemoglobin by more than 2 g/dl, *Before* before the administration of fondaparinux, *After* after the administration of fondaparinux. $^{**}p < 0.01$, $^{*}p < 0.05$

hemoglobin concentrations significantly decreased on days 4 and 8, thus suggesting that the decrease in hemoglobin was not due to the surgical procedure. The FDP, D-dimer, PPIC and SF levels were significantly higher in the anti-Xa group than in the without anti-Xa group, suggesting that a hyperfibrinolysis and hypercoagulable state exists after administration of fondaparinux. Fondaparinux cannot directly activate the fibrinolytic system, but it may increase secondary fibrinolysis, thus leading to increased bleeding. The increased bleeding causes a fibrin clot formation to activate fibrinolysis, followed by increases in the SF, FDP, D-dimer and PPIC levels, which all increase after treatment with fondaparinux. Therefore, fondaparinux may be useful for the treatment of DVT.

Eighteen of the 98 orthopedic patients who were treated with fondaparinux showed a reduction of more than 2 g/dl of hemoglobin (IB group). Total joint arthroplasty is associated with significant perioperative blood loss. The average range is from 761 to 1549 ml in a single TKA without antifibrinolytics [22–26]. There were no patients who stopped the fondaparinux treatment, and none of the patients required a blood transfusion. As the drainage tube was extracted before the injection of fondaparinux, the blood loss could not be measured. While there was no massive bleeding at the site of the operation after the administration of the anti-Xa inhibitor, there might have been some minor blood loss at the site. There are several causes of IB, such as surgery, hyperfibrinolysis, hemostasis in the host and anti-Xa agents. The current reports [22–26] suggest that as first suspected in this study, IB may be caused by elevated anti-Xa activity by fondaparinux. There was a wide range of anti-Xa activity in patients treated with fondaparinux, suggesting that high-dose administration of fondaparinux should be monitored by the anti-Xa activity. However, no significant difference was seen in the anti-Xa activity between the IB and non-IB groups before treatment and on days 1, 4 and 8 of fondaparinux administration. In addition, the anti-Xa activity was not markedly high in either group, thus suggesting that such increased bleeding may occur independent of the anti-Xa activity of fondaparinux. It might not be necessary to monitor anti-Xa activity following the injection of 1.5 mg fondaparinux.

The concentrations of FDP and D-dimer were significantly higher in the IB group than in the non-IB group before and on days 1 and 8, but there was no significant difference in the SF between these groups during that period. These findings suggest that clot formation and fibrinolysis before and on day 1 were higher in the IB than

Table 4 The plasma levels of PAI-I, SF and AT in the patients with and without a reduction in Hb >2.0 g/dl

	PAI-I (ng/ml)		SF (μg/ml)		AT (%)	
	IB+	IB−	IB+	IB−	IB+	IB−
Before	23.4 (21.2–26.3)	23.7 (19.2–35.0)	26.4 (19.7–71.0)	16.6 (10.7–38.3)	79.5 (68.5–84.1)	81.6 (73.4–89.8)
Day 1	19.5 (13.4–27.1)	16.7 (13.3–23.8)	10.6 (7.4–15.4)	13.3 (8.2–20.2)	78.5 (75.1–87.9)	84.1 (75.2–91.0)
Day 4	18.8 (13.0–23.1)	17.2 (12.8–23.1)	9.5 (7.0–17.3)	12.0 (8.7–17.3)	102.3 (86.8–104.8)	92.2 (84.0–103.1)
Day 8	15.0 (9.2–24.5)	15.7 (11.5–20.5)	5.2 (3.8–8.7)	7.1 (4.6–11.9)	104.9 (98.2–112.2)	99.9 (91.5–113.5)
Day 15			2.2 (1.9–4.5)	4.6 (3.2–8.6)	94.9 (91.3–105.9)	94.5 (87.8–104.8)

PAI-I plasminogen activator inhibitor-I, *SF* soluble fibrin, *AT* antithrombin, *IB* increased bleeding

Table 5 The correlation between the reduction of the hemoglobin concentration and hemostatic markers

rS Sperman's correlation coefficient

	rS			
	Day 1	Day 5	Day 8	Day 15
SF	0.157 (NS)	0.199 ($p < 0.05$)	0.144 (NS)	0.052 (NS)
D-dimer	0.082 (NS)	0.051 (NS)	0.405 ($p < 0.001$)	0.336 ($p < 0.001$)
FDP	0.115 (NS)	0.195 ($p < 0.05$)	0.390 ($p < 0.001$)	0.284 ($p < 0.01$)
PPIC	0.020 (NS)	0.388 ($p < 0.001$)	0.328 ($p < 0.001$)	

in the non-IB group. Therefore, bleeding due to surgery may affect the bleeding level from day 1 to 14.

D-dimer and FDP levels were again elevated at day 8 and those were significantly higher in the IB group in comparison to the non-IB group. Plasma levels of D-dimer, FDP and SF are elevated in patients with thrombosis [2–4], but D-dimer levels remain elevated long after the onset of DVT, but the levels of SF decrease rapidly [2], thus suggesting that the elevation of FDP and D-dimer indicates not only a thrombotic state, but also secondary fibrinolysis. These findings suggest that a hyperfibrinolysis instead of anti-coagulation may cause IB. The concentration of PPIC was significantly higher in the IB group in comparison to the non-IB group at day 0. The concentration of PPIC was highest on day 4, and the PAI-I level reduced at this point. There are many retrospective studies showing that tranexamic acid is useful for controlling blood loss after orthopedic surgery [22, 24]. Several prospective randomized double-blind studies [23, 27] also report that tranexamic acid significantly reduces blood loss after TKA.

In conclusion, the administration of 1.5 mg of fondaparinux was useful for the prevention of fatal PE and increased bleeding and fibrinolysis. Increased fibrinolysis and the use of an anti-Xa drug may, therefore, be associated with the IB group receiving thromboprophylaxis with fondaparinux following major orthopedic surgery. Future studies should therefore investigate the efficacy of 1.5 mg fondaparinux and anti-fibrinolytic therapy in patients with hyperfibrinolysis after orthopedic surgery.

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ORIGINAL ARTICLE

Frequent association of thrombophilia in cerebral venous sinus thrombosis

Makoto Ikejiri · Akihiro Shindo · Yuichiro Ii · Hidekazu Tomimoto · Norikazu Yamada · Takeshi Matsumoto · Yasunori Abe · Kaname Nakatani · Tsutomu Nobori · Hideo Wada

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Abstract Thrombophilia is frequently associated with venous thromboembolism (VTE) including cerebral venous sinus thrombosis (CVST). The possibility of thrombophilia was examined in 12 patients with CSVT diagnosed in the past 9 years. Thrombophilia due to abnormalities in antithrombin (AT), protein C (PC), or protein S (PS) or antiphospholipid syndrome (APS) was evaluated. Nine patients with abnormally decreased AT, PC or PS and one patient with APS were examined. Of the nine patients examined by a gene analysis of AT, PC, or PS, one had a congenital AT deficiency, one had a congenital PC deficiency, and two had congenital PS deficiencies including a novel mutant (Gly189Ala). AT, PC and PS levels were all decreased in one patient, PS level was decreased in three patients, and AT level was decreased in one patient at the onset of CVST, but these concentrations improved after treatment. CVST is frequently associated with thrombophilia and a transient decrease in AT, PC or PS may be a causal factor.

Keywords Cerebral venous sinus thrombosis (CVST) · Thrombophilia · Antithrombin · Protein C · Protein S

Introduction

There are approximately 170,000 new cases of clinically recognized venous thromboembolism (VTE) in patients treated in short-stay hospitals in the United States each year [1]. While cerebral venous sinus thrombosis (CVST) is a rare disease with an estimated annual incidence of 3–4 cases per 2 million adults, and 7 cases per 1 million neonates, its precise incidence is not known because there have been only a few epidemiological studies [2–4]. The risk factors for CVST are tumors [5], cerebral infections or trauma [5, 6], oral contraceptive use [7], pregnancy and the peripartum period [8] and thrombophilia [4, 7, 9]. Thrombophilia is defined as patients having a high risk for thrombosis, which can be either inherited or acquired. The main acquired thrombophilia is due to the presence of antiphospholipid antibodies [10, 11], and congenital thrombophilia includes antithrombin (AT), protein C (PC) or protein S (PS) abnormalities [12–14] and Factor V {G1691A} and Factor II (G20210A) abnormalities [15, 16]. However, Factor V {G1691A} and Factor II (G20210A) abnormalities have never been reported in Japan [17, 18].

The overall annual incidence of VTE was 1.53% in patients with deficiencies of AT, PC and PS [19] and thrombophilia has also been reported to be a risk factor for CVST [20].

M. Ikejiri · Y. Abe
Central Laboratory, Mie University Graduate School of Medicine, Tsu, Japan

M. Ikejiri · K. Nakatani · T. Nobori · H. Wada (✉)
Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan
e-mail: wadahide@clin.medic.mie-u.ac.jp

A. Shindo · Y. Ii · H. Tomimoto
Department of Neurology, Mie University Graduate School of Medicine, Tsu, Japan

N. Yamada
Department of Cardiology, Mie University Graduate School of Medicine, Tsu, Japan

T. Matsumoto
Department of Blood Transfusion, Mie University Graduate School of Medicine, Tsu, Japan

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In this study, we examined in 12 patients with CVST for thrombophilia due to AT, PC, and PS abnormalities and anti-phospholipid antibodies, and the expression of novel PS mutant was determined.

Materials and methods

Twelve patients with CVST were diagnosed at Mie University Hospital from 1 January 2003 to 28 February 2011 (Table 1). The CVST was diagnosed by magnetic resonance imaging (MRI), magnetic resonance venography (MRV) or cerebral angiography (CAG). The study protocol was approved by the Human Ethics Review Committee of the Mie University School of Medicine and a signed consent form was obtained from each subject. This study has been faithfully carried out in accordance with the Declaration of Helsinki. Case 1 was pregnant, case 3 had severe inflammation, case 5 had iron deficient anemia, and case 6 had been taking a contraceptive drug. Case 1 was complicated with deep vein thrombosis (DVT) and case 11 was associated with mesenteric venous thrombosis (MVT). No patients suffered from recurrent either CVTS or any other types of VTE after the 1st episode of CSVT.

Measurement of AT, PC, PS and antiphospholipid antibody concentrations

Peripheral blood samples were collected in a 1/10 volume of 3.13% sodium citrate. The plasma free PS antigen concentration was measured by a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) using an Asserachrom free PS kit (Diagnostica Stago, Asnières, France). The plasma PS and PC activity levels were measured by a clotting time method using a STA®-Staclot® Protein S and a STA®-Staclot® Protein C kit (Diagnostica Stago, respectively). The plasma PC antigen concentration was measured by a latex agglutination test using a LPIA-ACE PC (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The plasma AT activity was measured by a synthetic substrate assay using a Chromorate ATIII (C) kit (Mitsubishi Chemical Medience Corporation). An activity or antigen level of less than 70% in AT, PC and PS of the patients who did not receive warfarin treatment, was considered to be an abnormal decrease in AT, PC or PS. An abnormal decrease in PC or PS was defined as a >2-fold ratio of PS/PC or PC/PS activity in the patients who received warfarin treatment.

The dilute Russell's viper venom time (DRVVT) was measured by a clotting time method using a Gradipore LA test (Gradipore, Sydney, Australia). Anti-caldiolipin-β2 glycoprotein I (ACL-β2GPI) antibodies were measured with an ELISA kit (Yamasa Co, Tokyo, Japan).

Gene analysis of AT, PC and PS

Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. Each exon and exon/intron boundary of the gene was amplified from genomic DNA using the polymerase chain reaction (PCR) as described previously. The PCR products were directly sequenced using a Big-Dye Terminator Cycle Sequencing Kit and a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The transient and stable expression levels of recombinant Protein S and quantification of the expression level of PS were carried out as in a previous report [21]. The initiating Methionine was set to +1 and the amino acid residue of PS was numbered.

Statistical analysis

The differences in PS expression between mutant and wild type patients were determined using the unpaired t test. A value of $p < 0.05$ was considered to be significant.

Results

Decreased activity of AT was observed in 3 of the 12 patients (cases 1, 3, and 12) with CVST, decreased activity or antigen of PC was observed in 5 of the 12 patients (cases 3, 6, 7, 9, and 11), and decreased activity or antigen of PS was observed in 7 of the 12 patients (cases 2, 3, 4, 5, 6, 7, and 11), but 2 patients (cases 7 and 11) were already being treated with warfarin (Table 1). Case 8 was positive for both DRVVT and the ACL-β2GPI antibody, and was diagnosed as having antiphospholipid syndrome (APS). A gene analysis for AT, PC and PS was carried out, and 4 patients were diagnosed as having congenital thrombophilia: two with PS genetic abnormalities (cases 2 and 11), one with a PC genetic abnormality (case 9) and one with a AT genetic abnormality (case 1) (Table 2). Case 1 had a heterozygous Pro429Leu (AT Budapest) mutation of the AT gene. The pedigree of the family under investigation is shown in Fig. 1a. The proband developed CVST and DVT when she was pregnant. Her mother had the same mutation of AT and also had CVST. Her brother had no mutation, but the proband's child had same mutation. Case 2 had two heterozygous PS mutations (Asp79Tyr and Thr630Ile). Case 9 had a heterozygous Glu153 (458-460delAGG) mutation of PC. The pedigree of the family under investigation is shown Fig. 1b. The proband developed CVST at the age of 47 years, and her daughter was detected to have the same PC mutation. Therefore, prophylaxis for VTE could be performed for the daughter during her pregnancy.

Table 1 Subjects

Case	Age	Sex	Onset	CSVT	Additional factor	Other VTE	AT (%)	PC (%)		PS (%)		DRVVT (0.0–1.2)	ACL-β2GPI antibody
							Act (70–130)	Act (70–140)	Ag (70–130)	Ag (70–150)	Free Ag (70–150)		
1	27	F	2003	Straight sinus	Pregnancy	DVT	**55**	107	108	127	120	1.1	Negative
2	60	M	2010	Superior sagittal sinus	None	(–)	**88**	ND	96	**43**	**50**	ND	Negative
3	81	M	2010	Left sigmoid sinus	Inflammation, MOF	(–)	**64**	**44**	**31**	**34**	**69**	1.0	Negative
4	52	M	2007	Superior sagittal sinus Transverse sinus	None	(–)	109	115	112	**61**	**59**	ND	Negative
5	54	F	2005	Superior sagittal sinus	IDA	(–)	117	125	110	**65**	ND	0.9	Negative
6	40	F	2010	Superior sagittal sinus	Contraceptive use	(–)	88	**57**	76	**30**	**105**	1.2	Negative
7	57	M	2009, 2010	Superior sagittal sinus Transverse sinus	None	(–)	88	ND	65[a]	ND	52[a]	1.0	Negative
8	18	F	2006	Right transverse sinus	None	(–)	115	133	ND	73	ND	**1.3**	Positive
9	47	F	2008	Left transverse sinus	None	(–)	85	**59**	**60**	79	ND	1.0	Negative
10	49	M	2008	Superior sagittal sinus	None	(–)	98	96	99	103	97	ND	Negative
11	71	F	2009	Superior sagittal sinus	None	MVT	114	49[a]	55[a]	**18**[a]	45[a]	1.1	Negative
12	43	F	2011	Superior sagittal sinus	None	(–)	**65**	105	91	80	94	1.2	Negative

Values in parentheses show a reference. The bold values indicate no abnormal value

DVT deep vein thrombosis, *MVT* mesenteric venous thrombosis, *MOF* multiple organ failure, *F* female, *M* male, *IDA* iron deficient anemia, *Act* activity, *Ag* antigen, *ND* not done, *DRVVT* dilute Russell's viper venom time

[a] Treated with warfarin

Table 2 Possible hemostatic abnormalities due to CSVT

Case	Natural protease inhibitor deficiency or APS	Gene analysis
1	Congenital AT deficiency	Pro429Leu
2	Congenital PS deficiency	Asp79Tyr + Thr630Ile
8	APS	
9	Congenital PC deficiency	Deletion of 458-460 (AGG)
11	Congenital PS deficiency	Lys196Glu + Gly189Ala

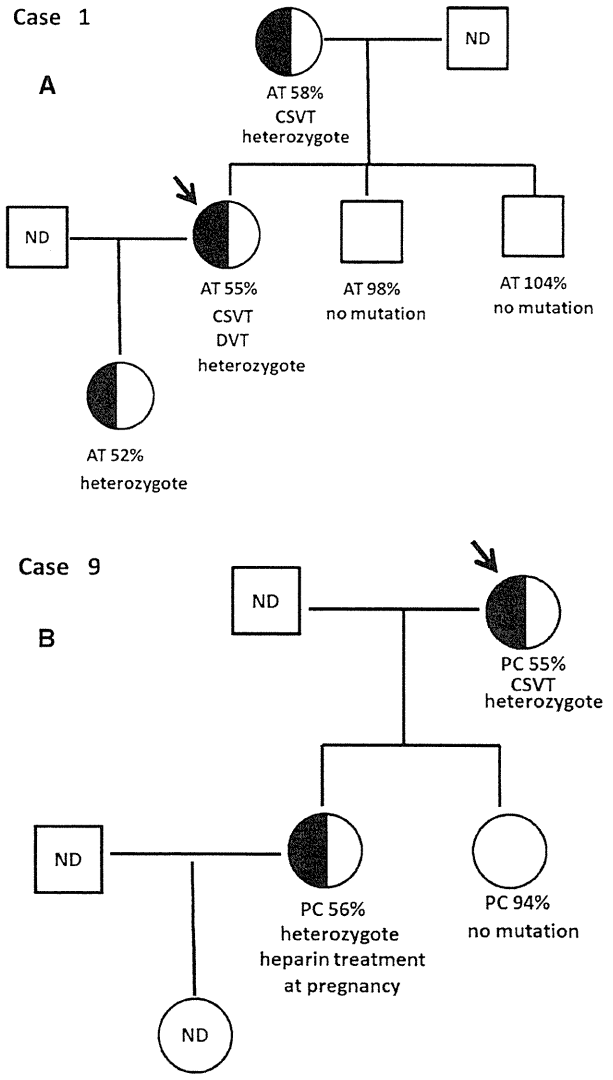

Fig. 1 The pedigree of the family under investigation. **a** Case 1, **b** case 9

Case 11 was a compound heterozygous mutation of the PS gene, including a novel mutation (Gly189Ala) and the PS Tokushima mutation (Lys196Glu). A PCR cloning strategy revealed that the Gly189Ala and the Lys196Glu mutations were in different alleles. This novel mutation was not detected in 100 healthy volunteers. Figure 2a shows the

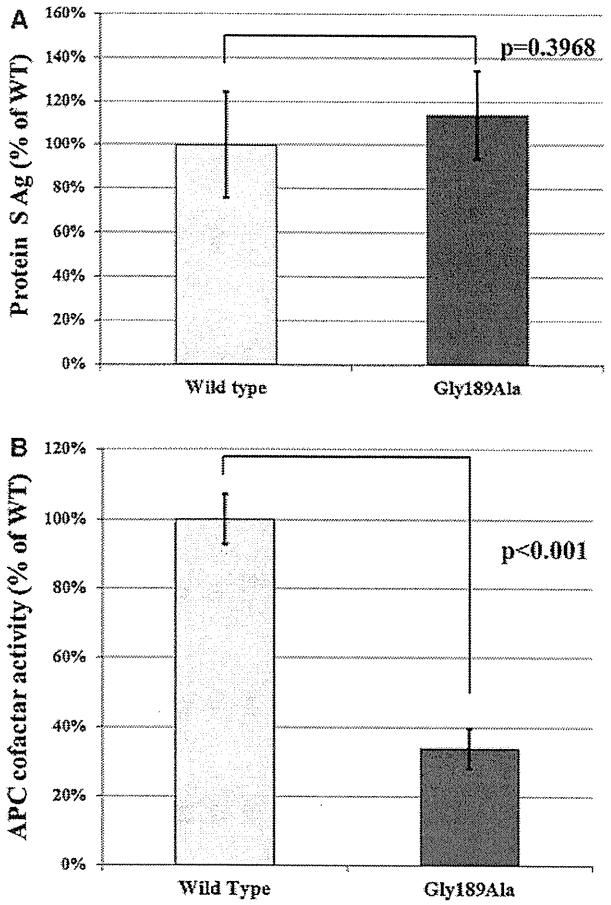

Fig. 2 The transient and stable expression of wild type and mutant recombinant PS in COS7 or BHK cells. **a** Transient expression in COS7 cells The determination of the concentration of PS in the conditioned media from genes containing different expressions using the ELISA method. The mean value of the wild type PS was defined as 100%. The *bars* represent the mean ± SD of six transfection experiments per each gene. The mutant and wild type expression levels were compared using unpaired *t* test. There was no significant difference. Wild type: 100.00 ± 24.26%, Gly189Ala: 113.70 ± 20.25%. **b** Stable expression in BHK cells. The APC cofactor activity was calculated using the clotting time and comparing it to the same concentration (100 ng/ml) of the different expressions levels. The mean value of wild type PS was defined to be 100%. The *bars* represent the mean ± SD of four experiments per gene. The mutant and wild type APC cofactor activity (100 ng/ml) was compared using unpaired *t* test. Wild: type; 100.00 ± 7.20%, Gly189Ala: 33.92 ± 5.81%

transient expression of wild type and mutant recombinant PS (Gly189Ala) in COS7 cells. The concentration of PS in the conditioned media from the different mutations was measured. No significant differences were observed between the mutant and wild type levels of antigen. Figure 2b shows the stable expression of wild type and mutant recombinant PS in baby hamster kidney (BHK) cells. The APC cofactor activity was significantly lower for the mutant PS (33.9 ± 5.8%) than for the wild type (100%, $p < 0.001$).

With regard to the onset of CVST, AT, PC and PS were all decreased in case 3, who had multiple organ failure, PS was decreased in cases 4, 5 and 6 and AT was decreased in case 12. After treatment for the CVST, these concentrations were improved, suggesting a transient AT, PC or PS deficiency. In addition, Case 7 had an abnormality in both PS and PC, but this patient was treated with warfarin. The ratio of PC/PS antigen was less than 2.0 and neither PC nor PS activity was measured. Therefore, case 7 was excluded from the gene analysis.

Discussion

CSVT is an uncommon condition with many clinical manifestations, so many cases remain clinically undetected. The incidence of severe thrombophilia due to AT, PC or PS deficiency and APS in CVST was reported to be 9% [4]. The odds ratio of the risk for CVST was reported to be 7.06 in patients with AT deficiency, 8.76 for PC deficiency, 3.20 for PS deficiency and 6.95 in patients with APS [20]. Although this study was of a small number of patients, the incidence of congenital thrombophilia was present in about 33.3% (4/12) of cases, thus suggesting that thrombophilia might be associated with CVST more frequently than was suggested in a previous report [4]. Because, a genetic analysis was not done in all cases with CVST of large-scale study [14].

In case 1, a patient with congenital AT deficiency had a heterozygous AT Pro429Leu (AT Budapest) mutation. This mutation is reported to lead to a decrease of heparin binding capability and protease inhibitor capability of the protein [22]. Case 2 had both PS Asp79Tyr and PS Thr630Ile mutations [21]. Asp79Tyr was previously reported as Asp38Tyr according to the previously established nomenclature system [21]. Asp79Tyr is a Type I PS deficiency (quantitative deficiency) that is characterized by a decrease in both the PS antigen levels and the PS activity [21]. We thought that the PS Asp79Tyr mutation was likely the cause of the decrease in PS, because it was previously reported that the PS Thr630Ile mutation does not influence the expression of PS [21]. Case 9 had a heterozygous PC Glu153del (458-460delAGG) mutation [23]. She experienced no complication during her two deliveries, but her daughter developed a hypercoagulable state during her pregnancy and was treated with low-dose heparin. In case 11, the patient had a PS Tokushima type II mutation characterized by a normal total and free PS antigen level, but a decrease in APC cofactor activity. This mutation is present in about 2% of the Japanese population [24–28]. The PS Gly189Ala is a novel mutation that we identified which was not a polymorphism in an analysis of 100 healthy volunteers [29]. The expression of the protein from this mutant is similar to the wild type protein, but the activity level is lower in comparison to wild type, thus suggesting this mutant to be a type II PS abnormality.

No mutations in AT, PC or PS were observed in the other 5 patients who did not receive warfarin treatment (Cases 3–6 and 12), although they had low AT, PC or PS at the onset of CVST. A decreased AT level was reported in patients with disseminated intravascular coagulation (DIC) [30] and liver diseases [31], decreased PC and PS levels were reported in patients treated with warfarin [32], and decreased PS was reported in pregnant females [33]. In case 3 who was in MOF status, the decreases in AT, PC and PS might have been caused by liver failure. The down regulation of the PS gene expression by 17β-estradiol, which increases in concentration in the late stages of pregnancy, has also been reported [34]. However, the decreased AT, PC or PS might be an outcome from thrombosis, rather than the cause of thrombosis in these patients. The mechanism(s) responsible for a decreased AT, PC or PS activity should be examined in future studies of various types of thrombosis, including CVST.

> "APS is one of the important causes of thrombosis [35], and APS is often observed in cases of cerebral infarction [36]. Most cerebral infarctions are considered to be due to arterial thrombosis, and these patients are usually treated with antiplatelet drugs, such as aspirin. CVST is usually recommended to be treated with warfarin. The differential diagnosis of CVST in patients with APS from other types of thrombosis due to arterial thrombosis is therefore important."

The frequency of thrombophilia is higher in CVST than in DVT, because CVST does not occur as frequently after surgery as DVT.

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Conflict of interest Authors have no direct or indirect conflict of interest in this manuscript

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Research Report

Gene expression associated with an enriched environment after transient focal ischemia

Yuji Shono[a], Chiaki Yokota[a,*], Yuji Kuge[b], Shinsuke Kido[c], Akina Harada[c], Koichi Kokame[d], Hiroyasu Inoue[e], Mariko Hotta[e], Kenji Hirata[f], Hideo Saji[c], Nagara Tamaki[f], Kazuo Minematsu[a]

[a]Department of Cerebrovascular Medicine, National Cerebral and Cardiovascular Center, Japan
[b]Central Institute of Isotope Science, Hokkaido University, Japan
[c]Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan
[d]Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center Research Institute, Japan
[e]Department of Food Science and Nutrition, Nara Women's University, Japan
[f]Department of Nuclear Medicine, Graduate School of Medicine, Hokkaido University, Japan

Article history:

Keywords:

* Corresponding author.

Abbreviations:

1. Introduction

2. Results

2.1. Infarct areas and recovery of the neurological functions

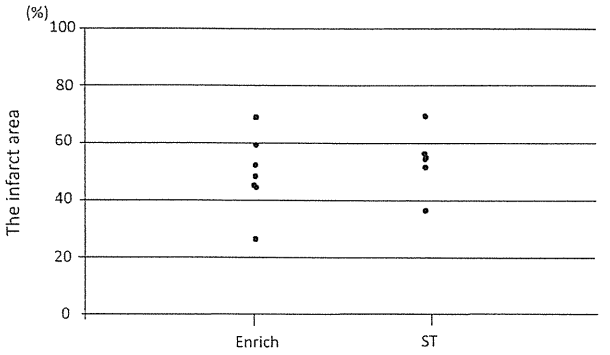

Fig. 1 – Comparison of infarct areas between enriched environment and standard cages. There were no significant differences in the infarct area between the enriched group (51.9% ± 9.4%) and the standard group (53.8% ± 10.6%).

2.2. cDNA array analysis

2.3. Gene expression associated with an enriched environment

3. Discussion