prospectively enrolled in the study. There were 136 patients with solid cancers (45: 91, 63.5 years old; 53.0 –72.0 years old), 94 with haematopoietic tumour (44: 50, 61.5 years old: 44.0 – 72.0), 78 with infectious disease (30: 48, 67.5 years old; 57.0 –72.0 years old), 31 with aneurysm (12: 19, 73.0 years old; 68.0 – 76.8 years old), 25 patients with trauma or burn (12: 13, 67.0 years old; 47.0 – 74.3 years old), 12 patients with cardiovascular disease (4: 8, 69.0 years old; (64.0 – 75.3 years old), 10 patients with gastrointestinal disease (7: 3, 58.0 years old; 22.0 – 63.0 years old), 8 with autoimmune disease (5: 3, 58.0 years old; 22.0 – 63.0 years old), 8 with obstetrical disease (8: 0, 33.0 years old; 30.5 – 34.5 years old) and 11 with other diseases (6: 5, 53.5 years old; 42.0 – 68.0 years old). The inclusion criteria were the observation of more than one abnormal finding according to the laboratory tests (platelet count; < 120/ $x10^3/\mu l$, FDP > 10 μg/ml, fibrinogen < 1g/l, PT ratio > 1.25) in addition to the presence of disease(s) associated with DIC. Any patients demonstrating associations with heparin-induced thrombocytopenia (HIT), thrombotic thrombocytopenic purpura (TTP), antiphospholipid syndrome (APS) or severe liver injuries were excluded. APS was diagnosed according to the Sapporo criteria (13), but one patient with symptoms of APS and antiphospholipid antibodies was excluded from this study after two months without undergoing any tests. Organ failure and inflammatory conditions were evaluated by the sepsis-related organ failure assessment (SOFA) (14) and the systemic inflammatory response syndrome (SIRS) score (15), respectively. 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 patient.

DIC was diagnosed on the day of registration using the JMHW, ISTH and JAAM diagnostic criteria (▶Table 1) (8). The onset of DIC within a week after the registration was defined as late-onset DIC. The DIC score using platelet count, FDP, fibrinogen and PT was thereafter checked in all patients not diagnosed with DIC every day after registration. Haemostatic molecular markers such as thrombin-AT complex (TAT), fibrin monomer complex (FMC), D-dimer, plasmin plasmin inhibitor complex (PPIC), thrombomodulin (TM) and AT were measured at registration. No DIC treatment was administered prior to the diagnosis of DIC.

PT, fibrinogen, platelet count and FDP were measured as previously described (16, 17). TAT, FMC, D-dimer, PPIC, TM and AT activity were measured by SRL Inc. (Tokyo, Japan). TAT and TM were measured by an enzyme immunoassay (EIA) using TAT [S] (TFB, Tokyo, Japan) and TM Banasera (Fujirebio, Tokyo, Japan), respectively. FMC, D-dimer and PPIC were measured by a latex immune agglutination (LIA) test using Auto LIA FM (Roche Diagnostic, Tokyo, Japan), LATECLE D-dimer (Kainos, Tokyo, Japan) and LPIA-ACE PPI II (Mitsubishi Chemical Medicine Corporation, Tokyo, Japan), respectively. AT activity was measured by means of heparin cofactor activity using the Testchyme S ATIII kit (Sekisui Medical, Tokyo, Japan).

Statistical analysis

The data are expressed as the median (25%-75% percentile). The differences between the groups were examined for statistical significance using the Mann-Whitney U test. A p-value < 0.05 was considered to be significant. A chi-square statistical analysis demonstrated an odds ratio (OR) of 95% confidence interval (CI) for the mortality, resolution rate from DIC, and the cut-off value of haemostatic parameters. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo, Japan).

Table 1: Three different diagnostic criteria for DIC established by the Japanese Ministry of Health and Welfare (JMHW), the International Society on Thrombosis and Haemostasis (ISTH), and the Japanese Association for Acute Medcine (JAAM).

Establish	Points	JMHW	ISTH	JAAM
Underlying disease	1	1 point	necessary	necessary
Clinical symptoms	1 1	bleeding* organ failure	-	SIRS 1 point
Platelet counts ($x10^3/\mu l$)	1 2 3	>80 but < 120 * >50 but < 80 * < 50*	>50 but < 100 < 50	>80 but < 120 #1 80< #2
Fibrin-related marker	1 2 3	FDP (μg/ml) >10 but < 20 >20 but < 40 >40	FDP, SF or D-dimer Moderately increased Markedly increased	>10 but < 25 >25
Fibrinogen (g/l)	1 2	>1 but < 1.5 <1	<1	-
PT, PT ratio, Prolongation of PT	1 2	>1.25 but <1.67 >1.67	Prolongation of PT >3 but 6<	>1.2
Diagnosis of DIC	points	≥7	≥5	≥4

JMHW, Japanese Ministry of Health and Welfare; ISTH, International Society on Thrombosis and Haemostasis; JAAM, Japanese Association for Acute Medcine. *: 0 points in patients with hematopoietic malignancy. #1: or a 30% reduction in the platelet count. #2: or a 50% reduction in the platelet count.

Table 2: Diagnostic rate according to three diagnostic criteria for DIC.

Underlying disease	JMHW	ISTH	JAAM
Solid cancer	47 (34.6%)	45 (33.1%)	95 (69.9%)
Haematopoietic tumor	39 (41.5%)	30 (31.9%)	71 (75.5%)
Infectious disease	36 (46.2%)	32 (41.0%)	60 (76.9%)
Aneurysm	15 (48.4%)	11 (35.5%)	22 (71.0%)
Trauma/Burn	7 (28.0%)	6 (24.0%)	19 (76.0%)
Cardiovascular disease	5 (41.7%)	6 (50.0%)	9 (75.0%)
Gastrointestinal disease	8 (80.0%)	7 (70.0%)	9 (90.0%)
Autoimmune disease	1 (12.5%)	0	5 (62.5%)
Obstetrics disease	6 (75.0%)	5 (62.5%)	5 (62.5%)
Other disease	2 (18.2%)	1 (9.1%)	5 (45.5%)
Total	166 (40.2%)	143 (34.6%)	291 (70.5%)

JMHW, Japanese Ministry of Health and Welfare; ISTH, International Society on Thrombosis and Haemostasis; JAAM; Japanese Association for Acute Medcine.

Results

Of the 413 patients, 166 (40.2%), 143 (34.6%) and 291 (70.5%) were diagnosed for DIC by the JMHW, ISTH and JAAM criteria, respectively (▶Table 2). The JAAM and ISTH overt-DIC diagnostic criteria diagnosed the highest and lowest numbers of patients, respectively. The high number of patients associated with DIC was evident for the cases of solid cancer, haematopoietic tumour and infectious disease. The prevalence of late onset of DIC was 12.1%, 13.3% and 13.9% using the JAAM, ISTH overt-DIC, and JAAM diagnostic criteria, respectively (▶Table 3). The mortality rate was 35.5%, 40.6% and 31.7% in the patients diagnosed using the JMHW, ISTH overt-DIC and JAAM diagnostic criteria, respectively. The sensitivity for death was the highest using the JAAM criteria (80.9%), and the specificity for death was the highest using the ISTH overt-DIC diagnostic criteria (71.4%). The OR for death (95% CI) was 1.88 (1.226|2.90, p< 0.005), 2.55 (1.65 – 3.95, p< 0.001) and 1.99 (1.196|3.32, p< 0.001) using the JMHW, ISTH overt-DIC and JAAM criteria, respectively.

Abnormalities of the global coagulation tests such as platelet count, PT ratio, FDP and fibrinogen were significantly higher in the patients with DIC than those without DIC using all three diagnostic criteria (▶Table 4). Platelet count was significantly lower in the DIC patients diagnosed using the JMHW criteria than in those patients diagnosed using the ISTH overt-DIC or JAAM criteria, and the platelet count was significantly higher in the patients without DIC diagnosed using the JAAM criteria than in those patients diagnosed using the ISTH overt-DIC diagnostic criteria. The PT ratio was significantly higher in the patients with DIC diagnosed using the ISTH overt-DIC diagnostic criteria than in those patients diagnosed using the JMHW criteria and JAAM criteria. The FDP was significantly lower in the patients without DIC diagnosed using the JAAM criteria than in those patients diagnosed using the ISTH-overt DIC criteria. The fibrinogen level was significantly higher in the patients with DIC diagnosed using the JAAM criteria than in those patients diagnosed using the JMHW and ISTH overt-DIC criteria.

The D-dimer, FMC, TAT, AT and TM abnormalities were significantly higher in the patients with DIC than those patients without DIC who were diagnosed using all three diagnostic criteria (▶Table 5). **There were no significant differences in D-dimer, FMC, TAT, PPIC, AT and TM levels of the patients with or without DIC diagnosed using the JMHW and ISTH criteria** (▶Table 5). The D-dimer, PPIC and AT levels abnormalities in the patients with DIC were significantly less using the JAAM criteria than using either the JMHW or ISTH criteria. The D-dimer, FMC, TAT and PPIC abnormalities in the patients without DIC were significantly less using the JAAM criteria than using the JMHW or ISTH criteria.

Discussion

Hitherto, the three diagnostic criteria for DIC have not been simultaneously evaluated. The present study prospectively evaluated the JMHW, ISTH and JAAM DIC diagnostic criteria in patients treated at Mie University Hospital and associated facilities. These diagnostic criteria use the same global coagulation tests but their

Table 3: Relationship between mortality and the diagnostic criteria.

	JMHW	ISTH	JAAM
DIC	166 (40.2%)	143 (34.6%)	291 (70.5%)
Without DIC	247	270	122
Late onset of DIC*	30 (12.1%)	36 (13.3%)	17 (13.9%)
Mortality in DIC	35.5% (59/166)	40.6% (58/143)	31.7% (92/291)
Sensitivity for death	51.3%	50.4%	80.0%
Specificity for death	64.9	71.4%	33.2%
Odds ratio for death	1.88 (1.22 – 2.90)	2.55(1.65 – 3.95)	1.99 (1.19 – 3.32)
	P< 0.005	P< 0.001	P< 0.001

Late onset of DIC: The patients were not diagnosed at registration but they were diagnosed to have DIC within one week.

Table 4: Global coagulation tests in the patients with DIC, those with late-onset DIC and those without DIC.

		JMHW	ISTH	JAAM
Platelet ($X10^4/\mu l$)	DIC(+)	& 4.3 (2.6~7.0)	& 6.3 (3.5~9.9)**	& 5.6 (3.2~7.7)*
	DIC(-)	& 9.6 (6.1~16.6)	& 9.4 (6.2~16.7)	& 15.5 (9.3~22.7)##
	Late	7.9 (5.8~12.4)	6.1 (3.0~8.3)*	12.6 (9.2~15.8)*##
PT ratio	DIC(+)	& 1.39 (1.16~0.76)	& 1.48 (1.28~1.95)*	& 1.27 (1.11~1.52)**##
	DIC(-)	& 1.12 (1.02~0.24)	& 1.12 (1.02~1.23)	& 1.08 (1.02~1.17)
	Late	1.21 (1.10~0.32)	1.18 (1.09~1.30)	1.19 (1.03~1.42)
FDP (μg/ml)	DIC(+)	& 43.0 (21.7~64.3)	& 38.0 (23.1~61.2)	& 31.9 (19.2~58.0)
	DIC(-)	& 20.1 (11.2~37.2)	& 20.2 (12.0~40.2)	& 16.4 (9.4~26.0)#
	Late	21.0 (16.7~30.7)	21.2 (15.1~42.7)	18.5 (10.4~21.8)
Fibrinogen (mg/dl)	DIC(+)	& 191 (120~345)	& 203 (115~334)	& 254 (150~365)*#
	DIC(-)	& 314 (236~398)	& 312 (212~397)	& 352 (245~446)
	Late	318 (192~378)	320 (191~370)	303 (125~382)

Data represent the median (25%tile – 75%tile). DIC(+): patients with DIC, DIC(-): patients without DIC, Late: patients with late onset DIC. && : $p< 0.01$ between DIC (+) and DIC (-). **, or *; $p< 0.01$ or $p< 0.05$ in comparison to DIC, without DIC or Late onset established by JMHW. ##, or #; $p< 0.01$ or $p< 0.05$ in comparison to DIC established by ISTH.

Table 5: Haemostatic molecular markers in the patients with DIC, those with late-onset DIC and those without DIC.

		JMHW	ISTH	JAAM
D-dimer (μg/ml)	DIC(+)	& 22.8 (11.9~45.0)	& 21.2 (11.2~38.3)	& 19.0 (9.7~35.6)*
	DIC(-)	& 10.3 (5.9~20.7)	& 12.0 (6.6~26.6)	& 8.6 (4.5~13.4)*##
	Late	21.3 (8.8~28.4)	17.3 (8.8~28.5)	16.1 (9.8~25.5)
FMC (μg/ml)	DIC(+)	& 112.0 (18.2~235.0)	& 110.0 (16.1~224.8)	& 70.8 (16.1~210.0)
	DIC(-)	& 33.0 (7.6~134.0)	& 36.6 (9.4~156.0)	& 16.7 (6.5~94.4)#
	Late	57.9 (14.0~151.3)	79.0 (12.3~182.0)	18.0 (8.9~79.6)
TAT (ng/ml)	DIC(+)	& 25.6 (13.3~90.0)	& 29.2 (13.2~90.0)	& 23.1 (10.8~62.8)
	DIC(-)	& 13.6 (6.8~32.3)	& 16.9 (7.5~40.9)	& 10.1 (5.1~28.0)#
	Late	18.1 (11.2~32.6)	17.3 (11.0~24.6)	18.9 (13.1~30.7)
PPIC (μg/ml)	DIC(+)	N 2.3 (1.0~6.8)	N 1.4 (0.9~6.2)	& 2.4 (1.2~6.2)#
	DIC(-)	S 2.0 (1.1~4.1)	S 2.3 (1.2~4.6)	& 1.6 (0.9~3.2)##
	Late	2.2 (1.4~3.6)	2.5 (1.4~5.7)	2.1 (1.1~2.6)
AT (%)	DIC(+)	& 63.9 (44.0~83.0)	& 53.6 (38.7~76.0)	& 68.0 (47.1~85.0)##
	DIC(-)	& 77.9 (56.0~99.8)	& 75.4 (55.7~95.3)	& 84.9 (59.5~102.0)
	Late	66.5 (51.7~83.0)	79.9 (61.1~89.8)	85.4 (62.0~120.0)
TM (ng/ml)	DIC(+)	& 5.3 (3.5~8.2)	& 5.6 (3.8~8.5)	& 4.9 (3.2~6.9)
	DIC(-)	& 3.9 (2.7~5.2)	& 4.2 (2.7~5.9)	& 3.6 (2.6~4.8)
	Late	4.1 (3.2~7.4)	4.0 (3.0~6.7)	4.3 (2.5~6.8)

Data represent the median (25%tile – 75%tile). Late onset: Late onset DIC. && or NS: $p< 0.01$, $p< 0.05$, or not significant between DIC (+) and DIC (-). **, or *; $p< 0.01$ or $p< 0.05$ in comparison to DIC established by JMHW. ##, or #; $p< 0.01$ or $p< 0.05$ in comparison to DIC established by ISTH.

cut-off values are different. The JAAM diagnostic criteria have been considered to have a high sensitivity for the diagnosis of DIC, and the ISTH overt-diagnostic criteria to have a high specificity for the diagnosis of DIC. The possibility of progression from the JAAM DIC to the ISTH DIC was reported (18). The latter study also reported the JMHW diagnostic criteria for DIC to be more sensitive than ISTH overt-DIC diagnostic criteria. A high number of associations with DIC were observed in cases of solid cancer, haematopoietic tumour and infectious disease. The frequency of DIC by the three diagnostic criteria in these underlying diseases was similar to that of the total patients, but the JMHW and ISTH criteria tended to display a low sensitivity for DIC in the patients with trauma and burn injuries. A late onset of DIC was observed in 13.9% of patients without DIC using the highly sensitive JAAM diagnostic criteria for DIC. This value was similar to that of the patients without DIC using either the JMHW criteria or ISTH criteria, thus suggesting that all of three diagnostic criteria might miss the early stage of DIC, since these criteria adopt same global coagulation tests which were not sensitive or specific for early stage of DIC. Haemostatic molecular markers such as TAT and SF might therefore be a sensitive indicator for the early phase of DIC (20).

The diagnostic criteria for DIC by JAAM, ISTH and JAAM were related to a poor outcome. In several trials of sepsis (6, 21), the patients associated with DIC displayed a poor outcome. In the pres-

What is known about this topic?

- There are three variations of diagnostic criteria for disseminated intravascular coagulation (DIC) established by the Japanese Ministry of Health and Welfare (JMHW), the International Society on Thrombosis and Haemostasis (ISTH) and the Japanese Association for Acute Medicine (JAAM).
- Three diagnostic criteria have been considered to be useful for the diagnosis of DIC.
- DIC patients are considered to have a poor outcome.

What does this paper add?

- Three diagnostic criteria were evaluated simultaneously.
- The JAAM diagnostic criteria have a high sensitivity for DIC and the ISTH diagnostic criteria have a high specificity for DIC.
- All three diagnostic criteria are related to a poor outcome.

ent study, the mortality of JAAM, ISTH and JAAM DIC was more than 30%. These data also proved the diagnosis of DIC by three diagnostic criteria to be related with a poor outcome. Furthermore, it is important to prove that DIC treatment improves the outcome of DIC and that the sensitivity of DIC diagnostic criteria for poor outcome is also important. The JAAM diagnostic criteria have the highest sensitivity, but the lowest specificity for poor outcome. Future studies should prospectively examine the effect of intervention for DIC treatment.

In this study all global coagulation tests such as platelet count, PT ratio, FDP and fibrinogen levels were significantly abnormal in the patients with DIC diagnosed by all three criteria, and these markers tended to be less abnormal in those patients with DIC who were diagnosed by JAAM criteria than in those patients diagnosed by the ISTH or JMHW diagnostic criteria. Of the haemostatic molecular markers, only PPIC was not useful for the diagnosis of DIC using all three diagnostic criteria. The observation that the D-dimer, FMC, TAT and AT markers also tended to be less abnormal in the patients without DIC who were diagnosed by the JAAM criteria, suggests that the JAAM criteria can detect mild haemostatic abnormalities. The values of TM and AT are reported to be worse in patients with poor outcome than in patients with a better outcome (22), thus suggesting that TM and AT may therefore be useful as markers of injured vascular endothelial cells. In the critical care field, a scoring system that includes the platelet count and PT has a prognostic value in severe sepsis (23).

In conclusion, all three diagnostic criteria for DIC are associated with a poor outcome and miss late-onset DIC at the time of admission. As a result, there are no useful markers for the late onset of DIC.

Acknowledgements

This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan for Blood Coagulation Abnormalities and from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Japanese Society of Thrombosis and Haemostasis, Japanese DIC Study Group.

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Int J Hematol (2011) 93:47–52
DOI 10.1007/s12185-010-0732-4

ORIGINAL ARTICLE

Elevated Von Willebrand factor propeptide for the diagnosis of thrombotic microangiopathy and for predicting a poor outcome

Naomi Ito-Habe · Hideo Wada · Takeshi Matsumoto · Kohshi Ohishi · Hidemi Toyoda · Eiji Ishikawa · Shinsuke Nomura · Yoshihiro Komada · Masaaki Ito · Tsutomu Nobori · Naoyuki Katayama

Received: 21 October 2010 / Revised: 15 November 2010 / Accepted: 18 November 2010 / Published online: 9 December 2010

Abstract Thrombotic microangiopathy (TMA) is associated with vascular endothelial cell injury and is sometimes linked with poor outcome. Von Willebrand factor (VWF) propeptide (VWFpp) is considered to be a marker of vascular endothelial cell injury. The plasma levels of VWF, VWFpp, and thrombomodulin (TM) were evaluated for their use in the diagnosis of TMA in 75 patients with TMA. There were 30 TMA patients with marked decreases in ADAMTS13 (TMA/ADAMTS13) and 45 without the decrease (TMA/other). The plasma levels of TM, VWF, and VWFpp values were significantly high in patients with TMA, especially TMA/other group. The plasma levels of TM and VWFpp were significantly high in non-survivor with TMA. In the TMA/other group, the plasma levels of VWFpp were negatively correlated with ADAMTS13 activity. The plasma levels of TM correlated with the renal function, but the plasma levels of VWFpp did not. A ROC analysis indicated that VWFpp and TM were useful markers for the prediction of a poor outcome. These findings suggest that VWFpp is an useful marker for the diagnosis of TMA and for the prediction of poor outcome.

Keywords VWFpp · TM · ADAMTS13 · TMA · Vascular endothelial cell injury

N. Ito-Habe · N. Katayama
Department of Hematology and Oncology, Mie University Graduate School of Medicine, Mie, Japan

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

T. Matsumoto · K. Ohishi
Transfusion Service, Mie University Hospital, Mie, Japan

H. Toyoda · Y. Komada
Department of Pediatrics, Mie University Graduate School of Medicine, Mie, Japan

E. Ishikawa · S. Nomura · M. Ito
Department of Cardiology and Nephrology, Mie University Graduate School of Medicine, Mie, Japan

1 Introduction

Thrombotic microangiopathies (TMAs) are defined by acute mechanical hemolytic anemia, thrombocytopenia, and visceral ischemic manifestations related to the formation of platelet thrombi in the microcirculation [1]. TMA includes thrombotic thrombocytopenic purpura (TTP), hemolytic ureic syndrome (HUS), hemolysis, elevated liver enzyme levels, low platelet (HELLP) syndrome, and complications after bone marrow transplantation. In addition, these symptoms show fluctuating bizarre neurologic symptoms, in addition to renal failure and fever [2, 3].

ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13), which was identified in 2001 [4–6], is a zinc metalloprotease that specifically cleaves unusually large Von Willebrand factor multimers (UL-VWFM) at the Tyr (1605)-Met(1606) boundary located in the A2 region of VWF [7, 8], suggesting that UL-VWFM cause multiple platelet thrombi due to TMA. Although the diagnosis of TMA has been improved remarkably by the development of a method for measuring ADAMTS13 [9, 10], some problems remain in the diagnosis of TMA without marked decrease of ADAMTS13.

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 [11]. 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 due to several physiological or pathological stimuli, VWFpp dissociates from VWF [12, 13].

Vascular endothelial cell injury is one of the main causes of and/or results of TMA and it has been reported that elevated thrombomodulin (TM) and VWF levels can be used as vascular endothelial cell injury markers in the patients with thrombotic thrombocytopenic purpura (TTP) and disseminated intravascular coagulation (DIC) [14, 15]. As elevated TM is observed in patients with renal failure, a more specific marker for vascular endothelial cell injury is required for the accurate diagnosis of TMA.

In this study, the plasma levels of TM, VWF, and VWFpp were measured in 140 patients with suspected TMA to evaluate the usefulness of diagnosis for TMA and for the prediction of a poor outcome.

2 Materials and methods

A total of 140 patients were suspected to have TMA and consulted us at Mie University Hospital between 1st January 1990 and 30th June 2010. There were 25 patients without underlying disease, 22 patients with autoimmune disease, 15 with malignant tumors, 12 who had undergone liver transplantation, 7 who had received born marrow or kidney transplantation, 14 with severe infection, 3 with O-157 infection, 4 due to pregnancy, 3 due to post-surgical complications, 2 due to drug use, and 33 patients with other diseases. Out of these patients, 75 were diagnosed to have TMA according to the diagnostic criteria of TMA: (1) thrombocytopenia (less than $12 \times 10^4/\mu l$), (2) hemolytic anemia (less than 11.0 g/dl of hemoglobin) due to the microangiopathy (presence of fragmented red cells, elevated total bilirubin, and LDH), (3) neurological dysfunction, (4) renal failure, and (5) fever [16]. The patients with (1) and (2) who had an ADAMTS 13 activity of less than 10%, who had an O-157 infection, and who had clinical symptoms, such as (3) or/and (4), were diagnosed with TMA.

The plasma levels of ADAMTS13 activity, thrombomodulin (TM), VWF, and VWFpp were measured in these patients and 50 healthy volunteers (19 females and 31 males; median age 31 years; range 19–51 years).

The ADAMTS13 activity was measured using a FRETS-VWF73, which was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) according to the method described by Kokame et al. [9, 10]. TM was measured with a Thrombomodulin "MKI" EIA kit (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). VWF and VWFpp levels were measured with a VWF&Propeptide assay kit (GTi DIAGNOSTiCs, Waukesha, USA).

These patients were classified into 3 groups; those with ADAMTS13-related TMA (TMA/ADAMTS13), where the ADAMTS13 level was less than 10%; TMA/other, the cause of which was not known; or non-TMA.

The study protocol was approved by the Human Ethics Review Committees of Mie University School of

Table 1 Characteristics of the TMA and non-TMA patients

	TMA/ADAMTS13	TMA/other	All-TMA	Non-TMA	All
Age; Median (25%tile–75%tile)	51.0 (34.8–67.3)	52.0 (33.8–61.8)	52.0 (34.5–64.5)	55.5 (38.0–71.0)	53.0 (36.0–67.0)
Sex (F:M)	17:13	30:15	47:28	33:32	80:60
Underlying disease					
Autoimmune disease	4	5	9	13	22
Malignant tumor	1	3	4	11	15
Liver transplantation	3	5	8	4	12
Other transplantation	0	3	3	4	7
Severe infection	3	8	11	3	14
O-157 infection	0	1	1	2	3
Pregnancy	1	3	4	0	4
Post-surgery	0	0	0	3	3
Drug use	0	1	1	1	2
Other	4	5	9	24	33
None	14	11	25	0	25
Non-survivors/all patients	5/30	16/45	21/75	7/65	28/140
Mortality (%)	16.7	35.6	28.0	10.8	20.0

Medicine, and signed informed consent was obtained from each patient.

2.1 Statistical analysis

The data are expressed as the medians (25% tile–75% tile). Differences between the groups were examined for significance using the Mann–Whitney *U* test for independence. A p value of less than 0.05 was considered to indicate a significant difference. Correlations between TM, VWF, VWFpp, and ADAMTS13 were examined using the Spearman's rank correlation coefficient. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo).

3 Results

The patient group included 30 with TMA/ADAMTS13, 45 with TMA/other, and 65 with non-TMA (Table 1). There were more female than male patients in the TMA/other group. Severe infection was the most frequent underlying disease in patients with TMA, while transplantation was the second leading cause, and autoimmune disease was the third leading cause of the disease. Severe infection was also the most frequent underlying disease in the TMA/other group. The mortality was 28.0% in the TMA, but 10.8% in the non-TMA group. The mortality tended to be higher in patients with TMA/other (35.6%) than TMA/ADAMTS13 (16.7%) (Table 1).

The median value (95% CI) of TM, VWF, and VWFpp in healthy volunteers were 15.2 (11.7–22.3) U/ml, 69.5 (33.0–170.0) U/dl, and 85.0 (39.5–160.3) U/dl, respectively (Table 2). The plasma levels of TM, VWF, and VWFpp values were significantly higher in non-TMA and all-TMA (TMA/ADAMTS13 and TMA/other) patients than in healthy volunteers ($p < 0.001$). The plasma levels of TM and VWFpp values were significantly higher in the all-TMA than in non-TMA patients ($p < 0.01$ and <0.001, respectively). Plasma levels of TM and VWFpp were significantly higher in the TMA/other [52.1 (31.8–69.2) U/ml and 279.0 (194.0–431.8) U/dl] than non-TMA patients [24.7 (17.3–35.0) U/ml and 171.0 (132.8–236.3) U/dl, $p < 0.001$, respectively], and in the TMA/other than the TMA/ADAMTS13 group [24.0 (19.4–33.9) U/ml and 196.0 (154.0–246.0) U/dl, $p < 0.001$ and <0.01, respectively, Table 2].

The plasma levels of TM in all patients ($p < 0.001$), all-TMA patients ($p < 0.001$), TMA/ADAMTS13 ($p < 0.05$), and TMA/other patients ($p < 0.05$) were significantly higher in non-survivors than survivors (Fig. 1). In addition, plasma levels of VWF in all patients ($p < 0.05$) and all-TMA ($p < 0.01$) were significantly higher in non-survivors than

Table 2 Thrombomodulin, Von Willebrand Factor, and Von Willebrand Factor propeptide values in control, non-TMA, and TMA groups

	Healthy volunteers median (95%CI)	Non-TMA median (25–75%tile)	All-TMA median (25–75%tile)	Non-TMA median (25–75%tile)	TMA/ADAMTS13 median (25–75%tile)	TMA/other median (25–75%tile)
TM (U/ml)	15.2 (11.7–22.3)	24.7*** (17.3–35.0)	35.7***, ## (21.2–59.1)	24.7* (17.3–35.0)	24.0*** (19.4–33.9)	52.1***, ###, +++ (31.8–69.2)
VWF (U/dl)	69.5 (33.0–170.0)	206.0*** (141.8–260.0)	191.0*** (142.3–265.8)	206.0* (141.8–260.0)	161.5***, # (109.0–206.0)	217.0***, ++ (149.3–281.3)
VWFpp (U/dl)	85.0 (39.5–160.3)	171.0*** (132.8–236.3)	233.0***, ### (166.5–341.3)	171.0* (132.8–236.3)	196.0*** (154.0–246.0)	279.0***, ###, ++ (194.0–431.8)

Data are expressed as the medians (95%CI) or median (25%–75%tile)

***, **, * $p < 0.001$, <0.01, or <0.05 in comparison to healthy volunteers; ###, ##, # $p < 0.001$, <0.01, or <0.05 in comparison to Non-TMA

+++, ++, + $p < 0.001$, <0.01, or <0.05 in comparison to TMA/ADAMTS13

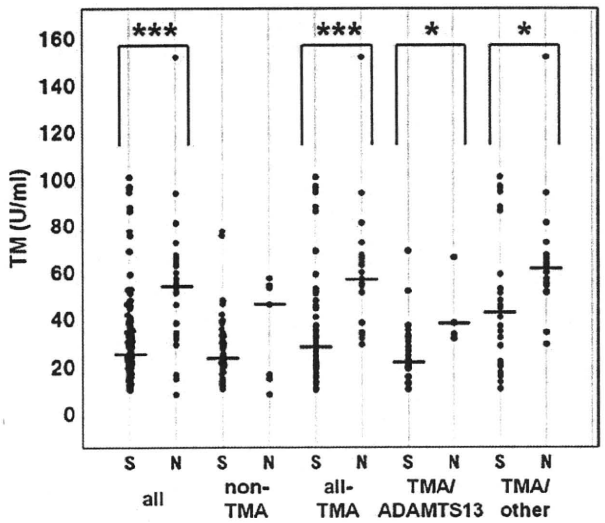

Fig. 1 Plasma levels of TM in the non-survivor and survivor groups. *S* survivor, *N* non-survivor. ***$p < 0.001$; *$p < 0.05$

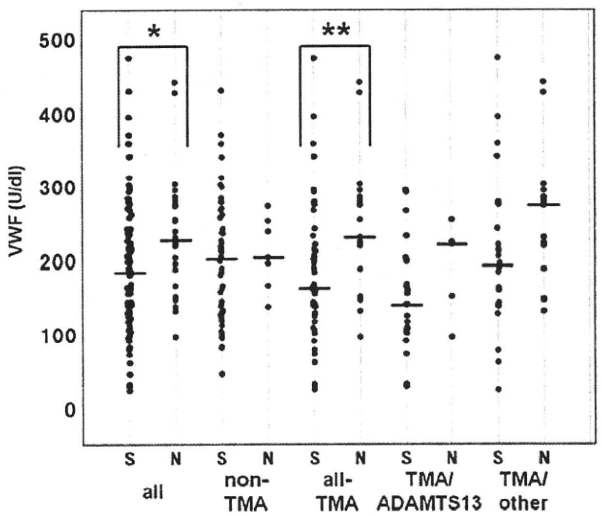

Fig. 2 Plasma levels of VWF in the non-survivor and survivor groups. *S* survivor, *N* non-survivor. **$p < 0.01$, *$p < 0.05$

survivors (Fig. 2). The plasma level of VWFpp in all patients (302.5 [230.5–457.5] U/dl vs. 182.0 [134.5–237.3] U/dl, $p < 0.001$), all-TMA (343.0 [278.5–510.0] U/dl vs. 201.0 [151.8–276.0] U/dl, $p < 0.001$), and TMA/other patients (436.5 [305.0–585.5] U/dl vs. 228.0 [152.0–308.0] U/dl, $p < 0.001$) were significantly higher in non-survivors than survivors (Fig. 3).

In the TMA/other group, the Spearman's rank correlation coefficient (r_S) with ADAMTS13 was −0.389 in TM, −0.298 in VWF, and −0.474 in VWFpp ($p < 0.01$, <0.05, and <0.01, respectively). There was a very low correlation of VWF and VWFpp with ADAMTS 13 activity in the all-TMA, TMA/ADAMTS13, and non-TMA groups. Plasma

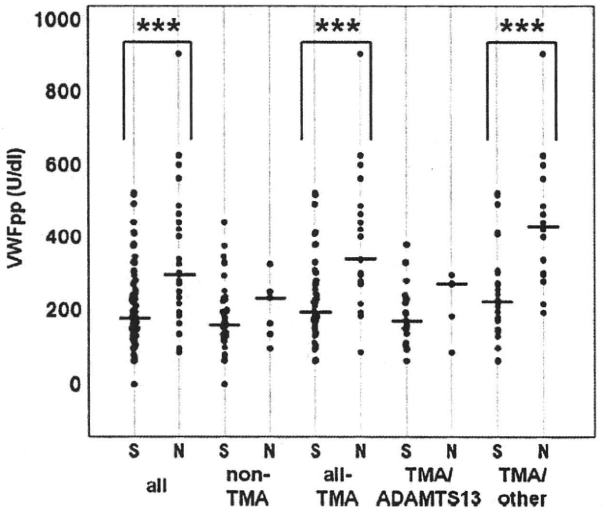

Fig. 3 Plasma levels of VWFpp in the non-survivor and survivor groups. *S* survivor, *N* non-survivor. ***$p < 0.001$

levels of TM in all patients were well correlated with E-glomerular filtration rate ($r_S = -0.734$, $p < 0.001$), but those of VWF ($r_S = -0.219$, NS), and VWFpp ($r_S = -0.261$, NS) were not.

In an ROC analysis of TM, VWF, and VWFpp for prediction of poor outcome, the area under the curve (AUC) was 0.783 for TM, 0.706 for VWF, and 0.796 for VWFpp in the all-TMA group, and the AUC was 0.716 for TM, 0.681 for VWF, and 0.825 for VWFpp in the TMA/other group (Fig. 4).

4 Discussion

In this study, the frequency of TMA/ADAMTS13 was 40% and the patients with an ADAMTS13 activity of less than 5% had an inhibitor for ADAMTS13. It was reported that a high titer of ADAMTS13 inhibitor has been reported to be related to a poor outcome [17]. In our cases, almost all patients with TMA/ADAMTS13 had a low titer of inhibitor demonstrated no relapse. The frequency of TMA/other was markedly high compared with national questionnaire survey done by the Japanese Ministry of Health, Labor and Welfare [18, 19] and other report [20]. As there are many reports of TMA due to abnormalities of ADAMTS13, most physicians look for a decrease in ADAMTS13 activity in patients with TMA. The high frequency of TMA/ADAMTS13 in the national questionnaire survey might have thus been caused by physician's bias. With regard to the diseases underlying the development of TMA, severe infection and transplantation were the most frequent in this study, but O-157 infection and autoimmune disease were the most frequent in the national questionnaire survey

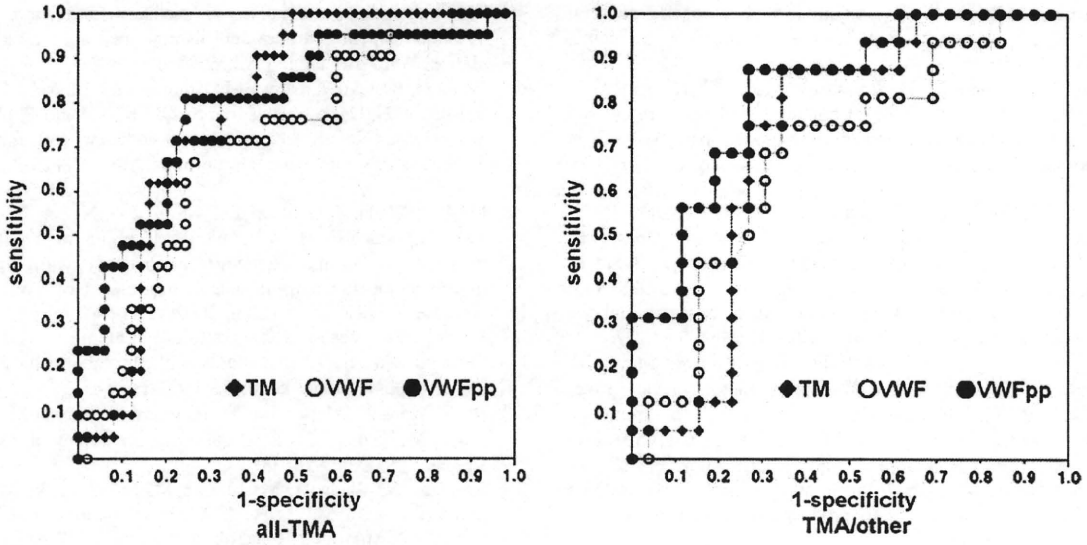

Fig. 4 Analysis of ROC for TM, VWF, and VWFpp in the prediction of poor outcome. The left side is all-TMA (TMA/ADAMTS13 + TMA/other) and right side is TMA/other. *Filled diamond* TM, *open circle* VWF, and *filled circle* VWFpp

[18, 19]. The deficiency of ADAMTS13 is a known cause of TMA, but the over-release of UL-VWFM from vascular endothelial cell may also be important. Severe infection often is associated with vascular endothelial cell injury and multiple organ failure. Auto-antibodies against ADAMTS13 were rarely detected in patients with malignant diseases or infections, and in those that were post-surgery or post-transplantation, all of which may cause TMA via vascular endothelial injuries and inflammation [21, 22].

The mortality of TMA in this study was 28.0%, which was slightly higher than in the national questionnaire survey [18, 19]. The mortality tended to be higher in TMA/other than in TMA/ADAMTS13 patients both in this study and in the national questionnaire survey. The high frequency of TMA/other in this study may have increased the mortality in comparison to the national questionnaire survey. These findings suggest that vascular endothelial cell injury may be related to poor outcome. Another study showed that a high titer of ADAMTS13 inhibitor may be related to poor outcome in Oklahoma study [17]. This finding suggests that a high titer of the inhibitor for ADAMTS13 may be related to the relapse of TMA. This discrepancy may be caused by the differences in the background of TMA. In analysis of TMA/other, VWFpp might be more useful for the prediction of poor outcome than TM.

The plasma levels of TM and VWFpp were significantly higher in the patients with TMA, especially TMA/other, thus suggesting that TMA might be associated with vascular endothelial cell injury and that elevated TM and VWFpp might be useful for the diagnosis of TMA/other. A contribution of acute endothelial dysfunction to renal impairment in sepsis is suggested by the significantly higher VWFpp and soluble TM levels in patients with increased creatinine values as well as by their strong positive correlations [23]. In contrast, the plasma levels of TM correlated with renal function, but those of VWFpp did not. In TMA/other patients, the VWFpp and TM levels were negatively correlated with ADAMTS13 activity, suggesting that vascular endothelial cell injury or the causes of vascular endothelial cell injury reduce the ADAMTS13 activity. In the event of severe sepsis, elastase derived from activated granulocyte might reduce the activity of ADAMTS13 [24].

In summary, there are many patients with TMA not due to markedly reduced ADAMTS13, and VWFpp may be useful for the diagnosis of this type of TMA.

Acknowledgments This work was supported in part by a Grant-in-Aid for Blood Coagulation Abnormalities from the Ministry of Health, Labor and Welfare of Japan. We thank Dr. Yoshihiro Fujimura and Dr Masanori Matsumoto of the Department of Blood Transfusion Medicine, Nara Medical University, Nara for the measurement of the inhibitor for ADAMTS13.

Conflict of interest All authors disclose no financial or personal relationship with other people or organizations that could inappropriately influence their work.

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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 INFO

Article history:
Accepted 17 December 2010
Available online 24 December 2010

Keywords:
Focal brain ischemia
Rat
Enriched environment
Functional recovery
Brain-derived neurotrophic factor
Early growth response

ABSTRACT

Recent studies have demonstrated that animals housed in an enriched environment after an experimental stroke obtained a better functional outcome than those housed in a standard cage; however, little is known about the gene expression associated with this functional recovery. The purpose of the present study was to elucidate the expression of genes in an enriched environment after experimental stroke in the ischemic and non-ischemic sides of the cortices. Transient focal brain ischemia was produced by the occlusion of the right middle cerebral artery (t-MCAO) in male Sprague–Dawley rats. The rats were divided into 3 groups: ischemic rats housed in the enriched environment, ischemic rats housed in standard cages, and non-ischemic rats in standard cages. Four weeks after t-MCAO, the rats were sacrificed and gene expression was examined. Motor function was improved in ischemic rats housed in the enriched environment compared with those in standard cages; however, there were no significant differences in the size of the infarct area between the ischemic rats in the enriched environment and those in standard cages. Decreases in the expression of Egr-1, -2, and BDNF mRNA in both sides of the cortices were detected in rats housed in the enriched environment, indicating that gene expression was altered throughout the brain at 4 weeks after transient focal ischemia.

* *Corresponding author.* Department of Cerebrovascular Medicine, National Cerebral and Cardiovascular Center, 5-7-1, Fujishirodai, Suita, Osaka 565-8565, Japan. Fax: +81 6 6872 7486.
E-mail address: cyokota@hsp.ncvc.go.jp (C. Yokota).

Abbreviations: t-MCAO, transient middle cerebral artery occlusion; NSS, Neurological Severity Score; MAP-2, anti-microtubule-associated protein 2; BDNF, brain-derived neurotrophic factor; Egr, early growth response

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doi:10.1016/j.brainres.2010.12.058

1. Introduction

Stroke is the major worldwide cause of mortality and morbidity. Motor exercise is essential for functional recovery after stroke. Forced exercise, for example, treadmill running or constraint-induced movement therapy, has been shown to enhance the functional recovery of motor skills after experimental ischemic stroke (Kim et al., 2005; Kim et al., 2009; Taub et al., 2002); however, other studies demonstrated that treadmill running produced negative physiological adaptations induced by stress (Moraska et al., 2000) and a constraint-induced movement study did not improve functional outcome after brain ischemia (Muller et al., 2008). Therefore, the effect of forced exercise on functional recovery after stroke is controversial. On the other hand, an enriched environment, which induces social interactions, perceptive stimuli, and voluntary exercise, has recently been demonstrated to positively influence the outcome after experimental brain damage. Several studies demonstrated that animals housed in an enriched environment after ischemic stroke obtained a better functional outcome as compared with those housed in standard cage (Biernaskie and Corbett, 2001; Nygren and Wieloch, 2005; Ohlsson and Johansson, 1995). Gene expression under an enriched environment was examined in the hippocampus and sensorimotor cortex after experimental brain injury using microarray techniques (Keyvani et al., 2004; Ronnback et al., 2005). These studies indicated that an enriched environment affected the mRNA expression levels of microtubule-associated protein, synapse-related molecule, neurotransmitter receptors, neuroprotective factors, and so forth. However, one study could not confirm the gene changes identified from the microarray analysis using real-time PCR (Ronnback et al., 2005), and this study only evaluated the mRNA expression levels of the non-ischemic side. Another study demonstrated that an enriched environment induced similar changes of mRNA expression in normal and ischemic brains (Keyvani et al., 2004). However, these studies did not show functional recovery after brain injury and it remains unclear which factors affect gene expression more profoundly, the enriched environment or the ischemic brain injury itself. Limited evidence exists for gene expression associated with functional recovery after focal brain ischemia under an enriched environment. The purpose of the present study was to elucidate the expression of genes under an enriched environment after experimental stroke in the ischemic and non-ischemic sides of the cortices.

2. Results

2.1. *Infarct areas and recovery of the neurological functions*

The infarct area evaluated by immunoreactivity to MAP-2 in the enriched group (51.9%±9.4%) was not significantly different to that in the standard group (53.8%±10.6%) (Fig. 1).

The neurological functions of the rats in both ischemic groups gradually improved after the first evaluation (Fig. 2). There were no significant differences in the NSS at 2 and 4 weeks after the first evaluation between the enriched and standard groups. For motor function evaluated by the inclined plane test in 2 positions, the rats housed in an enriched environment showed a significantly better recovery rate compared with those housed in standard cages at 4 weeks after t-MCAO (the third evaluation).

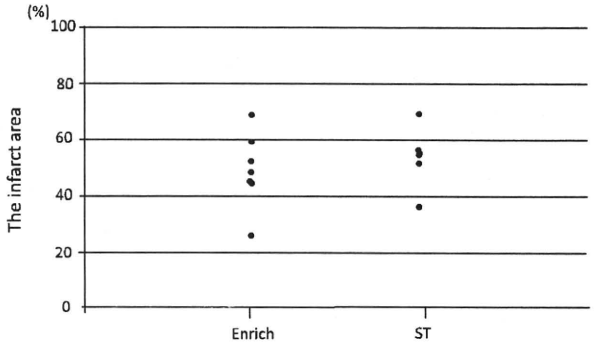

Fig. 1 – Comparison of infarct areas between enriched environment and standard cages. There were no significant differences in the infarct area between the enriched group (51.9%±9.4%) and the standard group (53.8%±10.6%).

2.2. *cDNA array analysis*

The expression of nectin-3 gene was increased (1.31-fold) and the expression of brain-derived neurotrophic factor (BDNF) (0.84-fold) and early growth response (Egr)-1 (0.68-fold) and -2 (0.68-fold) was decreased in the enriched group as compared with the standard group.

2.3. *Gene expression associated with an enriched environment*

There were no significant differences in mRNA expression of those 4 genes between the ischemic and non-ischemic sides (Fig. 3). In the non-ischemic side (left), BDNF and Egr-1 mRNA levels were significantly decreased in the enriched group compared with the standard and normal groups. Egr-2 mRNA was significantly increased in the normal group compared with enriched and standard groups. Comparing the mRNA expression of the ischemic side (right) among the three groups, BDNF mRNA was significantly decreased in the enriched and standard groups compared with the normal group. Egr-1 mRNA was significantly decreased in the enriched group compared with the standard and normal groups. Egr-2 mRNA was significantly decreased in the enriched group compared with the normal group. Nectin-3 mRNA expression among the 3 groups was not significantly different in each side.

3. Discussion

We examined gene expression associated with an enriched environment after transient focal ischemia. Motor function improved in ischemic rats housed in the enriched environment compared with those in standard cages; however, there

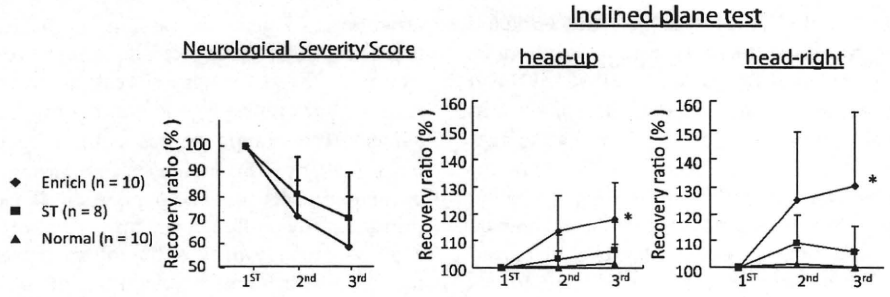

Fig. 2 – Changes of recovery after transient focal ischemia. There were no significant differences in the neurological severity score at 2 (the 2nd evaluation) and 4 weeks (the 3rd evaluation) after transient focal ischemia between the enriched and standard groups. To evaluate motor function, the inclined plane test was performed with the head in 2 different orientations: head-up and head-right. The rats housed in the enriched environment showed significantly better recovery compared with those in standard cages at 4 weeks (the 3rd evaluation) after transient focal ischemia. *$p<0.05$; Enrich, enriched group; ST, standard group; Normal, normal group; 1st, the 1st evaluation; 2nd, the 2nd evaluation; and 3rd, the 3rd evaluation.

were no significant differences in the size of the infarct area at 4 weeks after brain ischemia between the ischemic rats in the enriched environment and those in standard cages. These findings were consistent with previous studies (Biernaskie and Corbett, 2001; Nygren and Wieloch, 2005; Ohlsson and Johansson, 1995), suggesting that the motor recovery enhanced by the enriched environment was not a result of the reduction of infarct volume. One of the explanations of this finding would be due to the bilateral neuronal plasticity induced after the focal ischemic insult. We demonstrated that alterations of gene expression occurred not only in the ischemic side but also in the non-ischemic side. Biernaskie and Corbett (2001) showed the evidence that enhanced dendric complexity and length were confirmed in ischemic enrichment animals compared with both ischemic standard and sham animals. Clinical studies support the concept of functional reorganization after stroke (Cramer et al., 1997; Weiller et al., 1993).

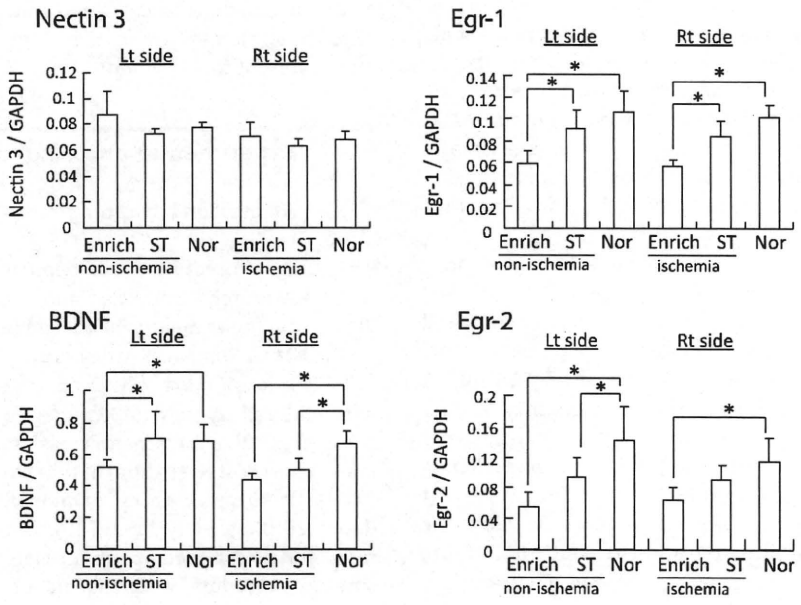

Fig. 3 – Comparison of gene expression in the whole brain among the 3 groups: ischemic rats in the enriched environment or standard cages, and non-ischemic rats. There were no significant differences in the mRNA expression of the four genes between the right and left sides. There were also no significant differences in nectin-3 mRNA expression for any condition. Egr-1 mRNA on both sides and BDNF mRNA on the left side levels were significantly decreased in the enriched group compared with the standard or normal groups. BDNF mRNA levels on the right side and Egr-2 mRNA levels on the left side were significantly decreased in the enriched and standard groups compared with the normal group. Egr-2 mRNA levels on the right side were significantly decreased in the enriched group compared with the normal group. *$p<0.05$; Enrich, enriched group; ST, standard group; Nor, normal group; Lt, left; and Rt, right.

The main finding of this study was that the enriched environment after transient focal ischemia resulted in decreases in the expression of Egr-1, -2, and BDNF mRNA in both sides of the cortices in rats. The expression of Egr-1 in both sides decreased more significantly in ischemic rats housed in the enriched environment compared with those in standard cages. There were no significant differences in the expression of the Egr-1 gene between the ischemic rats housed in standard cages and non-ischemic rats. The increased expression of Egr-1 was detected between 2 h and 5 days with a peak increase of 8–12-fold at 1 day after the transient focal brain ischemia in rats (Tureyen et al., 2008). They also indicated that Egr-1 induction significantly contributes to post-ischemic inflammation and secondary brain damage. As the gene expression analyses were performed at 4 weeks after the transient focal ischemia in the present study, the level of Egr-1 expression would therefore have decreased to the level observed in the normal rats. Egr-1 was previously shown to be a master switch for the initiation of inflammatory gene expression under ischemic stress (Yan et al., 2000). The decreased expression of Egr-1 in the rats housed in the enriched environment compared with those in standard cages in the present study reflects the suppression of post-ischemic inflammation in the whole brain of the ischemic rats housed in the enriched environment, although the underlying mechanisms are unclear. The expression of intracellular adhesion molecule-1 (ICAM-1) gene was decreased in the enriched group as compared with the standard group (0.68-fold) in the one time of the DNA array analysis in the present study. ICAM-1, which was known to be an endothelial cell–leukocyte adhesion receptor on endothelial cell (Diamond et al., 1990) and also to mediate transmigration (Lawrence et al., 1990), was induced in brain microvessels in the ischemic zone after focal cerebral ischemia and reperfusion in a primate model (Okada et al., 1994). Decreased expression of ICAM-1 gene in the enriched group could reflect the suppression of post-ischemic inflammation. On the other hand, little is known about the specific role of Egr-2 in the central nervous system.

The expression of BDNF was decreased in the non-ischemic (left) side of the brain in the ischemic rats housed in the enriched environment as compared to ischemic and normal rats housed in standard cages in the present study. Zhao et al. (2000) reported that the expression of BDNF mRNA 2–12 days after permanent focal brain ischemia in rats housed in an enriched environment was significantly lower in the ipsilateral and contralateral cortices to the ischemic lesion than in those housed in standard cages. In the present study, the expression of BDNF mRNA 4 weeks after the transient focal ischemia in the non-ischemic side of the enriched environment group was decreased compared to normal as well as standard rats; however, there were no significant differences in the expression of BDNF gene between the enriched environment and standard cages in the ischemic side. These inconsistencies of the present study would be attributable to the differences of severity of ischemic insult due to the different animal model from the previous study. Zhao et al. also showed decreased BDNF expression compared with standard cages in the bilateral hippocampus after permanent focal ischemia, and these alterations of gene expression in the hippocampus were more prominent than those in bilateral cortices. Because we examined the gene expression by the use of the whole brain tissue, alterations of gene expressions of BDNF and other genes in the hippocampus cannot be confirmed in the present study. The increased expression of BDNF was not observed in both sides of the cortices in ischemic rats in the present study, although the up-regulation of BDNF has been suggested to play an essential role in neuronal plasticity after focal brain ischemia (Ploughman et al., 2005; Ploughman et al., 2007). One explanation for these findings may be the differences in exercise administered to the ischemic rats. Although we tried to promote an endurance exercise by rearranging the location of objects in cage twice a week in the enriched environment group, the intensity as well as the duration of exercise would be different from previous studies (Ploughman et al., 2005; Ploughman et al., 2007). Another explanation could be due to the possibility of the suppression of post-ischemic inflammation in the enriched environment reflected by decreased expression of Egr-1 and ICAM-1 genes. The delay in the preparation of brain tissue after transient focal ischemia could be also leading a cause of decrease in the BDNF expression.

4. Conclusion

Motor function was improved in rats housed in the enriched environment. Decreases in the gene expression of Egr-1, -2, and BDNF in the rats housed in the enriched environment in both sides of the cortices could indicate that alterations in gene expression are induced in the whole brain at 4 weeks after transient focal ischemia.

5. Experimental procedures

5.1. *Animals and surgical procedures*

All animal procedures were approved by our Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council. Male Sprague–Dawley rats, 9 weeks old and weighing 270–320 g, were used in this study. Rats were anesthetized with chloral hydrate (400 mg/kg body weight i.p.), and transient focal brain ischemia was produced by the intraluminal occlusion of the ostium of the right middle cerebral artery (MCA) for 60 min with nylon monofilaments, as previously described (Kuge et al., 1995). Rectal temperature was monitored and maintained at approximately 37 °C throughout the surgical procedure with the aid of heating pads. Normal controls did not undergo the sham operation.

5.2. *Housing conditions and neurological evaluations*

In the ischemic animals, neurological disorders were measured using the Neurological Severity Score (NSS), a composite of motor, sensory, reflex, and balance tests (Chen et al., 2001). The NSS was graded on a scale of 0–18, and 1 score point was added for a failure in each exam. All rats were confirmed to

Table 1 – Results of the first neurological evaluation.

Neurological evaluation	Enrich	Standard	Control
Neurological Severity Score	8.5±1.4	8.0±1.1	0.2±0.4*,**
Inclined plane test (angle)			
Head-up	40.0±4.8	42.2±3.1	48.4±2.9*,**
Head-right	36.4±7.0	42.4±2.8	49.9±2.8*,**
			(mean±SD)

Enrich indicates enriched environment group; ST, standard group.
* $p<0.05$ vs. Enrich
** $p<0.05$ vs. ST

have no neurological deficits before transient MCA occlusion (t-MCAO). Evaluation of NSS was examined 72–96 h after t-MCAO at the first evaluation. To compare rats of almost equal neuronal damage, only the rats that scored 7–12 at the first evaluation were used for the subsequent examinations (Table 1). After the first evaluation, rats were housed in an enriched environment (enriched group; $n=10$) or standard cages (standard group; $n=8$). The normal control group (control group; $n=10$) was housed in standard cages. The second evaluation was done at day 14 and the third was done at day 28 after divided into groups. The enriched group was housed in larger cages (610×460×460 mm), and 4–6 rats were housed together. The enriched cage contained various toys, for example, a running wheel, tunnel, etc., and the location of objects in cage including toys, food, and water were rearranged twice a week. The size of the standard cage was 320×210×130 mm, and the standard and control groups were separately housed in each cage. All groups were housed under a 12-h light/dark cycle.

To evaluate the motor deficits, we performed the inclined plane test at the same time of measuring NSS. All rats were confirmed not to have any motor deficits before t-MCAO. Each rat was initially placed on a stainless steel plane inclined at a 30° angle. The angle of the inclined plane was then increased at a rate of 2°/s, and we measured the angle at which the rat's limbs slipped. The inclined plane test was performed with the head in two different orientations: head-up and head-right. For the presentation of the NSS and inclined plane test results, we calculated the mean value of the recovery ratio of the condition at 2 and 4 weeks after t-MCAO to that of the first evaluation for each group. There were no significant differences of neurological function between the enriched environment and standard groups in the first evaluation (Table 1).

5.3. *Brain preparation and histological examinations*

Four weeks after the rats were divided into the three groups, brain tissues were perfused with cold saline, and the animals were sacrificed by exsanguination under chloral hydrate anesthesia. Brains were then cut into 3 coronal sections at 3 mm intervals from the frontal pole. The first blocks (peri-infarct area) and the third blocks (peri-infarct and infarct core areas) were frozen in isopentane–dry ice and stored at −80 °C for gene analysis, and the second blocks (peri-infarct and infarct core areas) were embedded in paraffin for histological analysis. We used the first and second blocks in the present study.

The second blocks were stained by immunohistochemistry using an anti-microtubule-associated protein 2 (MAP-2), a neuronal skeletal protein, antibody. Deparaffinized cortical sections (5 μm) were preincubated with 10% normal pig serum (Kohjin Bio Co., Ltd.). The sections were incubated with the monoclonal anti-MAP-2 antibody (1:400; Pharmingen) overnight at 4 °C. The following day, the sections were rinsed and incubated with biotin-conjugated pig IgG (1:500; Dako) and peroxidase-conjugated streptavidin (1:500; Dako) for 30 min and visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. Immunoreactivities to MAP-2 were measured using VHX-100 (KEYENCE). The infarct area was calculated as follows: infarct area (%) = (MAP-2 staining area in intact side − MAP-2 staining area in ischemic side)/MAP-2 staining area in the intact side × 100.

5.4. *Gene analysis*

cDNA array analysis was conducted using the Rat Genome 230.2.0 Array and GeneChip Instrument System (Affymetrix) containing approximately 28,000 genes. Poly(A)$^+$-RNA extracted from the first blocks in the ischemic side from the enriched group ($n=3$) were pooled and those from 3 animals in the standard group were also pooled. The normalized gene expression signals were compared between the 2 groups. We performed cDNA array analysis for 2 times in this study. For cDNA array analysis, we selected those genes with increases in their normalized gene expression signals above 1.1-fold or decreases below 0.9-fold both 2 times and whose function was known.

5.5. *Real-time quantitative reverse transcriptase–polymerase chain reaction (real-time RT–PCR)*

Real-time quantitative reverse transcriptase–polymerase chain reaction (real-time RT–PCR) was used to confirm the changes in gene expression identified using cDNA array analysis. Eighteen samples were obtained from the bilateral cortices of the first blocks in nine animals (enriched group, $n=3$; standard group, $n=3$; and normal group, $n=3$). Total RNA from the samples was isolated using the acid guanidinium thiocyanate procedure and analyzed for the expression of the 4 identified genes (BDNF, nectin-3, Egr-1, and -2) using real-time RT–PCR (Mx3005; Stratagene). After converting total RNA (5 μg) to cDNA using SuperScript III reverse

Table 2 – Oligonucleotide primers used in real-time quantitative RT–PCR.

Gene	Sequences (forward primer) (Reverse primer)
Nectin 3	TGGTTTATTGGCGTCAGACA (f958)
	GATCCTGGACGTCAGCAGTT (r1119)
BDNF	AGGACGCGGACTTGTACACT (f930)
	GCTGTGACCCACTCGCTAAT (r1115)
Egr-1	AACAACCCTACGAGCACCTG (f549)
	AGGCCACTGACTAGGCTGAA (r750)
Egr-2	AGCTGCCTGACAGCCTCTAC (f402)
	GTTTCTAGGCGCAGAGATGG (r604)

transcriptase (Invitrogen), quantitative PCR was performed using SYBR Green Realtime PCR Master Mix Plus (Toyobo, Japan). The sequence of the primer pairs for each of the genes and their cycling number (N) are described in Table 2. The cycling conditions were 3 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 67 °C for 30 s. The expression levels of each mRNA were normalized to those of GAPDH mRNA.

5.6. *Data analysis*

A Student's t test was used to analyze differences in the infarct areas between the enriched and standard groups and gene expression between the hemispheres in each group. A Tukey–Kramer test was used to evaluate the recovery ratio of neurological functions. A one-way analysis of variance (ANOVA) followed by Fisher's post-hoc test was used to assess the differences in hemispheric gene expression among the 3 groups. A two-tailed p-value of <0.05 was considered to be significant. Data are expressed as the mean ± standard deviation.

Acknowledgments

This study was supported in part by a grant from the Mitsubishi Pharma Research Foundation, by research grants for cardiovascular diseases (21A-4, 22-4-1) from the Ministry of Health, Labor, and Welfare of Japan, and by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science.

Disclosures

None.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 18, pp. 13444–13453, April 30, 2010

Down-regulation of *PROS1* Gene Expression by 17β-Estradiol via Estrogen Receptor α (ERα)-Sp1 Interaction Recruiting Receptor-interacting Protein 140 and the Corepressor-HDAC3 Complex*

Received for publication, September 4, 2009, and in revised form, February 26, 2010 Published, JBC Papers in Press, March 3, 2010, DOI 10.1074/jbc.M109.062430

Atsuo Suzuki[‡], Naomi Sanda[§], Yuhri Miyawaki[‡], Yuta Fujimori[‡], Takayuki Yamada[‡], Akira Takagi[‡¶], Takashi Murate[‡¶], Hidehiko Saito[‖], and Tetsuhito Kojima[‡¶1]

From the [‡]Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya 461-8673, Japan, [§]Department of Clinical Laboratory, Nagoya University Hospital, Nagoya 466-8550, Japan, [¶]Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya 461-8673, Japan, and [‖]Nagoya Central Hospital, Nagoya 453-0801, Japan

Pregnant women show a low level of protein S (PS) in plasma, which is known to be a risk for deep venous thrombosis. 17β-Estradiol (E_2), an estrogen that increases in concentration in the late stages of pregnancy, regulates the expression of various genes via the estrogen receptor (ER). Here, we investigated the molecular mechanisms behind the reduction in PS levels caused by E_2 in HepG2-ERα cells, which stably express ERα, and also the genomic ER signaling pathway, which modulates the ligand-dependent repression of the PSα gene (*PROS1*). We observed that E_2 repressed the production of mRNA and antigen of PS. A luciferase reporter assay revealed that E_2 down-regulated *PROS1* promoter activity and that this E_2-dependent repression disappeared upon the deletion or mutation of two adjacent GC-rich motifs in the promoter. An electrophoretic mobility shift assay and DNA pulldown assay revealed that the GC-rich motifs were associated with Sp1, Sp3, and ERα. In a chromatin immunoprecipitation assay, we found ERα-Sp protein-promoter interaction involved in the E_2-dependent repression of *PROS1* transcription. Furthermore, we demonstrated that E_2 treatment recruited RIP140 and the NCoR-SMRT-HDAC3 complex to the *PROS1* promoter, which hypoacetylated chromatin. Taken together, this suggested that E_2 might repress *PROS1* transcription depending upon ERα-Sp1 recruiting transcriptional repressors in HepG2-ERα cells and, consequently, that high levels of E_2 leading to reduced levels of plasma PS would be a risk for deep venous thrombosis in pregnant women.

Protein S (PS)[2] is a vitamin K-dependent plasma protein that functions as a nonenzymatic cofactor for activated protein C in the down-regulation of the blood coagulation cascade via proteolytic inactivation of coagulant factors Va and VIIIa (1). PS has been also shown to display activated protein C-independent anticoagulant activity in purified systems as well as in plasma (2, 3).

Over the past 2 decades, low levels of plasma PS have become a well established risk factor for the development of deep venous thrombosis (4–6). Hereditary PS deficiency has been shown to be an autosomal dominant trait, and many causative genetic mutations have been described in the PSα gene (*PROS1*) (7). However, PS deficiency can also occur throughout life under acquired conditions such as oral anticoagulant use and liver disease (8). Furthermore, acquired PS deficiency has been reported in individuals with high levels of estrogen during pregnancy and in those taking oral contraception (9–11).

The major source of circulating plasma PS is the hepatocyte (12), but PS is also produced constitutively at low levels by a variety of other cell types throughout the body (13–17). PS circulates in human plasma at a concentration of 0.35 μM in a free form (40%) and a C4b-binding protein-bound form (60%) (18, 19). Two copies of the PS gene located on chromosome 3, the active PSα gene (*PROS1*) and the inactive PSβ pseudogene (*PROS2*), share 96% homology in their coding sequences (20–22). The promoter and first exon are absent from the *PROS2* gene, and the promoter region of *PROS1* has been poorly investigated in contrast to the coding regions. It has been reported that transcription from the *PROS1* promoter is directed from multiple start sites and that the *PROS1* 5′-flanking region lacks the characteristic "CAAT" and "TATA" boxes (23).

Estrogens are important regulators of mammalian growth and metabolism, accomplishing these functions by controlling the expression of specific genes via estrogen receptors (24). The estrogen receptor (ER) is a member of the steroid/nuclear receptor superfamily of transcription factors and is required for the mediation of 17β-estradiol (E_2)-induced responses in multiple tissues and organs (25). The classical ER mechanism of

* This study was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology (19590553), the Ministry of Health and Welfare (Health Sciences Research Grant, Research on Specific Disease), and the Kitamura Memorial Foundation, Japan.

[1] To whom correspondence should be addressed: Nagoya University School of Health Sciences, 1-1-20, Daiko-Minami, Higashi-ku, Nagoya 461-8673, Japan. Tel. and Fax: 81-52-719-3153; E-mail: kojima@met.nagoya-u.ac.jp.

[2] The abbreviations used are: PS, protein S; ChIP, chromatin immunoprecipitation; CS-FBS, charcoal-stripped FBS; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; FBS, fetal bovine serum; HDAC, histone deacetylase; LCoR, ligand-dependent corepressor; NCoR, nuclear receptor corepressor; re-IP, re-immunoprecipitation; RIP140, receptor-interacting protein 140; RT, reverse transcription; siRNA, small interfering RNA; iNS, nonspecific siRNA; SMRT, silencing mediator of retinoid and thyroid hormone receptors; Sp, specificity protein; TSA, trichostatin A; WT, wild type.

action involves ligand-induced formation of an ER homodimer that interacts with estrogen-responsive elements (EREs) in target gene promoters and recruits cofactors necessary for transactivation (25). There is increasing evidence that the formation of a classical genomic ER-ERE complex is only one of several genomic and non-genomic pathways of estrogen actions (26–28). Genomic ER associates with other transcription factors such as the activating protein-1 (AP-1) complex, nuclear factor κB (NFκB), and specificity proteins (Sp) to modulate ligand-dependent gene expression (26, 27, 29). In this study, we investigated the molecular mechanisms of the reduction in PS production caused by E_2 as well as the genomic ER signaling pathway that modulates ligand-dependent *PROS1* gene repression in HepG2-ERα cells stably expressing human ERα.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human hepatoma cell line HepG2 and human breast cancer cell line MCF7 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Tokyo) supplemented with 5 or 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and 100× antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan). Human normal hepatocytes (hNhep®) were purchased from Lonza (Walkersville, MD) and cultured in collagen I-coated dishes using the HCM™ BulletKit® according to the manufacturer's protocols. For estrogen assays, cells were cultured in phenol red-free DMEM (Invitrogen) supplemented with 10% charcoal-stripped FBS (CS-FBS) for 3 days. Next day, the cells were cultured in phenol red-free DMEM supplemented with 1% CS-FBS and treated with ethanol (vehicle) or 100 nM E_2 for 48 h. E_2 and trichostatin A (TSA) were purchased from Sigma-Aldrich, and ICI 182,780 was from Tocris Bioscience (Ellisville, MO).

Luciferase Constructs of PROS1 Promoter—First, we isolated a 14-kb BamHI fragment containing the *PROS1* promoter from a human genomic library (Clontech, Mountain View, CA). We used a PCR-amplified DNA probe (−582 to +173 with A of the ATG translation initiation codon as +1), which is the 5′-flanking region of human *PROS1* including a part of exon 1, preventing *PROS2* detection. The library was screened with a DIG-High Prime DNA labeling and detection kit (Roche Applied Science) according to the manufacturer's directions. An XbaI/SalI fragment (−4229 to +117) from the isolated *PROS1* gene was subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA) and removed from −3 to +117 by BstBI/SalI digestion and self-ligation. Subsequently, the *PROS1* −4229/−4 fragment was cloned into a pGL3 Basic-derived vector (gift from Dr. Kokame (30)) (pPROS1/−4229). A series of deletion constructs (pPROS1/−1826, pPROS1/−1119, pPROS1/−582, pPROS1/−338, pPROS1/−236, and pPROS1/−175) were obtained by digestion with the respective restriction enzymes and self-ligation or by PCR amplification. Mutated constructs (pPROS1/−175Mut1, pPROS1/−175Mut2, and pPROS1/−175Mut3) were prepared by mismatch PCR as described elsewhere (31) using the following forward primers (mutated bases are underlined): 5′-CCGAGCTCTTACGCGTGGGAGCGAACGGTCTCCTC-3′ (Mut1), 5′-CCGAGCTCTTACGCGTGGGAGCGGGCGGTCTCCTCCGAACCCGGC-3′ (Mut2), and 5′-CCGAGCTCTTACGCGTGGGAGCGAACGGTCTCCTCCGAACCCGGC-3′ (Mut3). pPROS1/−4229Mut was constructed from pPROS1/−4229 by the QuikChange Lightning site-directed mutagenesis kit (Stratagene) according to the manufacture's instructions using the following primers: 5′-GGAGCGAACGGTCTCCTCCGAACCCGGCTG-3′ and 5′-GCCGGGTTCGGAGGAGACCGTTCGCTCCCA-3′.

ERα Expression Plasmid and Stable Transfectants—Human ERα cDNA was obtained by reverse transcription (RT)-PCR from MCF7 mRNA. We amplified the cDNA as two fragments using as primers 5′-GGGAATTCTTTCTGAGCCTTCTGCCCTG-3′/5′-CATGTCGAAGATCTCCACCATG-3′ for upstream fragments (1347 bp) and 5′-ACCGAAGAGGAGGGAGAATGT-3′/5′-GGCTCGAGCTTGGAATCCCTTTGGCTGT-3′ for downstream fragments (1232 bp). The upstream and downstream PCR products were digested with EcoRI/HindIII and HindIII/XhoI, respectively, and ligated as a full-length human ERα cDNA into the pcDNA3.1 vector (Invitrogen) (pERα).

pERα was transfected by a calcium precipitation method as described previously (32), and stable transfectants were established from HepG2 cells through selection with G418. All clones were checked by Western blot analysis as described below, and the subclone showing the highest level of ERα (HepG2-ERα) was used for further study.

Enzyme-linked Immunosorbent Assay for Measurement of PS—HepG2-ERα cells were treated with E_2 or vehicle (ethanol only) for 48 h, and the culture medium was harvested. A rabbit IgG against human PS (Dako, Carpinteria, CA) was biotinylated with an ECL™ protein biotinylation module (Amersham Biosciences). An unlabeled anti-PS polyclonal antibody (2.4 ng/100 μl) in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 3 mM NaN_3) was coated onto each well of a microtiter plate (Nunc, Roskilde, Denmark). After three washes with 150 μl of Tris-buffered saline (TBS; 50 mM Tris, pH 7.4, 150 mM NaCl), the wells were blocked with 1% bovine serum albumin in TBS and then incubated with the plasma standards or culture medium samples. After three more washes with TBS, the biotinylated anti-PS antibody (0.1 μg/100 μl) was added followed by diluted (1:1000) streptavidin-horseradish peroxidase conjugate (Amersham Biosciences). After incubation, the substrate buffer (0.65 mg/ml *o*-phenylenediamine (Wako) and 0.06% H_2O_2 in 0.1 M citrate, 0.2 M sodium phosphate buffer, pH 5.0) was added to each well. After further incubation at room temperature for 20 min, the peroxidase reaction was stopped by the addition of 50 μl of 2 M H_2SO_4, and absorbance was measured at 490 nm.

Quantitative RT-PCR for Measurement of PS mRNA—Total RNA of the cells was extracted using an RNeasy mini kit (Qiagen, GmbH, Germany). The first-strand cDNA was prepared with 5 μg of total RNA using the SuperScript III first strand system (Invitrogen). Quantitative RT-PCR was performed with a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and ABI PRISM 7000 sequence detection systems (Applied Biosystems) were used for measurement. Relative PS mRNA was calculated as the respective PS

mRNA/GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA as described previously (33).

Western Blot Analysis—Proteins were extracted from the cultured cells by harvesting in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 850 mM 2-mercaptoethanol, 5% glycerol, and 0.001% bromphenol blue). Samples were resolved by 10% SDS-PAGE and transferred to Immonilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with excess protein (2% skim milk) and probed with primary antibody (1:1000) against ERα, Sp1, Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (Cytoskeleton Inc., Denver, CO). After being washed with phosphate-buffered saline containing 0.05% Tween 20, membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology, Danvers, MA) for 1 h. Signals were visualized with a chemiluminescent substrate (ECL Plus Western blotting detection system, Amersham Biosciences).

Luciferase Reporter Assay—Cells were seeded in 35-mm dishes at a concentration of 1.0×10^5 cells in phenol red-free DMEM supplemented with 10% CS-FBS. After 18 h, the appropriate *PROS1* luciferase reporter plasmids (3 μg) and pSV-β-galactosidase plasmid (0.2 μg; Promega, Madison, WI) were transiently co-transfected using Lipofectin® reagent (Invitrogen) according to the manufacturer's protocol.

After a 6-h transfection, the cells were washed and treated for 48 h with fresh phenol red-free DMEM supplemented with 1% CS-FBS containing 100 nM E_2, 100 nM E_2/1 mM ICI 182,780, 1 mM ICI 182,780 only dissolved in ethanol, or ethanol alone as a vehicle control. The cells were harvested, and subsequently luciferase activity was determined with a luciferase assay system (Promega) according to the manufacturer's directions. Luciferase activity was normalized to the activity of co-transfected β-galactosidase as an internal control for transfection efficiency.

Transient Transfection of siRNA—HepG2-ERα cells were cultured in phenol red-free DMEM with 10% CS-FBS and transfected with siRNA against Sp1 or Sp3 (Ambion, Austin, TX) or nonspecific siRNA using Oligofectamine reagent (Invitrogen) according to the manufacturer's directions.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from HepG2-ERα cells using NE-PER® nuclear and cytoplasmic extraction reagents (Pierce) and stored in aliquots at −80 °C until further use. The protein concentration of the nuclear extracts was measured with the Bio-Rad protein assay kit (Bio-Rad). DNA probes containing the *PROS1* promoter fragment (from −176 to −147) were synthesized, biotinylated, and annealed. EMSA was performed according to a method described previously (32). Briefly, nuclear extract (5 μg) and a biotin-labeled double-stranded DNA probe (600 fmol), with or without an unlabeled competitor, were treated with a LightShift™ chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions. In supershift experiments, the nuclear extract was incubated on ice for 10 min with the biotin-labeled double-stranded DNA probe after which an anti-Sp1, anti-Sp3, or anti-ERα antibody was added, and the incubation was continued for another 20 min. Samples were loaded on a 6% nondenaturing polyacrylamide gel in 0.5× TBE buffer (0.089 M Tris borate, pH 8.0, 0.089 M boric acid, and 10 mM EDTA) and electrophoresed for 3.5 h at 100 V. Biotin-labeled DNA probes were transferred to Hybond™-N+ membranes (Amersham Biosciences) and then integrated with streptoavidin-horseradish peroxidase conjugate.

DNA Pulldown Assay—The DNA pulldown assay (DNA affinity precipitation; DNAP assay) was carried out with biotin-labeled DNA probes as described previously (34). The nuclear extracts (100 μg) were prepared from HepG2-ERα cells and incubated with biotin-labeled DNA probes (100 pmol) and 15 μg of polydI-dC in DNAP buffer (20 mM HEPES-KOH, pH 7.9, 80 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 0.1% Triton X-100) on ice for 45 min. Subsequently, 500 μg of Dynabeads® M-280 streptavidin (Invitrogen) was added and incubated further at 4 °C for 1 h. The beads were washed three times with DNAP buffer, and the bound proteins were eluted in SDS sample buffer, separated by 10% SDS-PAGE, and characterized by Western blot analysis with the respective specific antibodies.

Chromatin Immunoprecipitation (ChIP) and ChIP Reimmunoprecipitation (Re-IP) Assays—HepG2-ERα cells were treated with E_2 or vehicle and fixed with 2% formaldehyde. The cross-linking reaction was stopped by the addition of 0.125 M glycine. After two washes with phosphate-buffered saline, the cells were resuspended in a swelling buffer (10 mM Tris-HCl, pH 7.6, 3 mM $CaCl_2$, 0.1% Nonidet P-40, and 1× protease inhibitor mixture (Nakalai Tesque)). After incubation on ice for 10 min, the samples were mixed by vortex, and the nuclei were collected. Isolated nuclei were resuspended in SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, and 1× protease inhibitor mixture) and sonicated to the desired chromatin length (0.5 kb). After centrifugation, the supernatant was diluted with dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, and 1.1% Triton X-100) containing 1× protease inhibitor mixture, divided into aliquots, and precleared by the addition of protein A-agarose or protein G PLUS-agarose (Santa Cruz Biotechnology). In the ChIP assay, the precleared chromatin supernatants were immunoprecipitated with the respective antibodies specific to ERα, Sp1, Sp3, nuclear receptor corepressor (NCoR), silencing mediator of retinoid and thyroid hormone receptors (SMRT), histone deacetylase 1 (HDAC1), HDAC3, HDAC4, HDAC5, receptor-interacting protein 140 (RIP140), ligand-dependent corepressor (LCoR) (Santa Cruz Biotechnology), and AcH4 (Millipore) or nonspecific IgG at 4 °C overnight. The protein-antibody complexes were incubated with protein A-agarose or protein G PLUS-agarose at 4 °C overnight. The beads were washed extensively in the following buffers: low salt wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and 1× protease inhibitor mixture); high salt wash buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and 1× protease inhibitor); and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunocomplexes were extracted from the beads with elution buffer (0.1 M $NaOH_3$ and 1% SDS) and reversed by heating at 65 °C overnight. Bound DNA was purified with a High Pure PCR cleanup micro kit (Roche Applied Science) and used as a template for subsequent amplification. The primers were 5′-

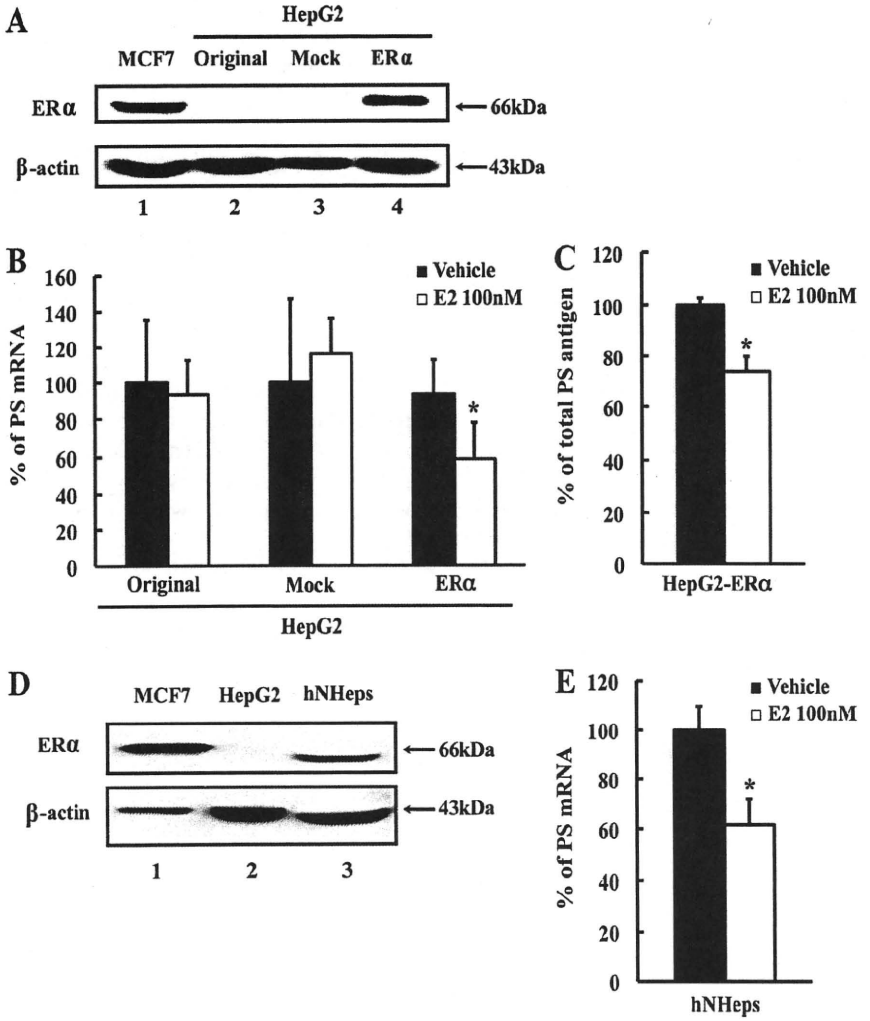

FIGURE 1. **Down-regulation of PS mRNA and antigen in HepG2-ERα cells and human normal hepatocytes by 17β-estradiol.** *A*, analysis of whole cell extract from MCF7 cells used as a positive control (*lane 1*), the original HepG2 cells (*lane 2*), mock-transfected HepG2 cells (*lane 3*), and HepG2-ERα cells (*lane 4*) by Western blotting using anti-ERα antibody. Anti-β-actin antibody was used as a loading control. *B* and *C*, results of quantitative RT-PCR and an enzyme-linked immunosorbent assay to evaluate the effects of E_2 on the levels of PS mRNA in HepG2-ERα cells and its derived cells (*B*) and on the levels of PS antigen secreted from HepG2-ERα cells (*C*). After treatment of HepG2-ERα cells with E_2 for 48 h, total RNA and the culture medium were harvested and analyzed by quantitative RT-PCR and an enzyme-linked immunosorbent assay, respectively. Values are the mean ± S.D. for at least three independent experiments. *, $p < 0.05$ *versus* vehicle control. *D*, analysis of whole cell extract from MCF7 cells used as positive control (*lane 1*), the original HepG2 cells used as negative control (*lane 2*), and hNHep cells (*lane 3*) by Western blotting using anti-ERα antibody. Anti-β-actin antibody was used as a loading control. *E*, results of quantitative RT-PCR to evaluate the effects of E_2 in hNHep.

GCTCCGAAAAGCTTCCTGGAA-3′ (−236/−217, forward) and 5′-CGCCTCGGTCTGAGCCGT-3′ (−88/−105, reverse), which amplified a 149-bp region of the *PROS1* promoter containing target GC-rich motifs. The primers amplifying a 159-bp region of the *PROS1* promoter that contained no GC-rich motif were 5′-GGAGAATGAGGGGCAAGA-3′ (−4033/−4016, forward) and 5′-CATTTCATCACCTTAGCAACCT-3′ (−3875/−3896, reverse), and those amplifying a 175-bp region of the *PROS1* promoter containing a non-target GC-rich motif were 5′-AGGAGAGCAGGGCAGGATAA-3′ (−3748/−3729, forward) and 5′-GGACAGAAGCCCAATCATAGTAAAT-3′ (−3574/−3598, reverse). PCR products were resolved on a 2% agarose gel in the presence of 1 μg/ml ethidium bromide.

In the ChIP re-IP assay, the precleared chromatin supernatants were immunoprecipitated with the first antibody, anti-ERα or anti-RIP140, at 4 °C for overnight. The protein-antibody complexes were incubated with protein G PLUS-agarose at 4 °C for 2–4 h, eluted by incubation with 10 mM dithiothreitol at 37 °C for 30 min, and diluted 1:50 in dilution buffer. After centrifugation, the supernatants were divided in aliquots and reimmunoprecipitated with their respective second antibodies individually. The second immunocomplexes were extracted from the beads followed by PCR amplifications of a 149-bp region of the *PROS1* promoter containing target GC-rich motifs from bound DNA as described above.

Statistical Analysis—Data are presented as the mean ± S.D. and are representative of at least three independent experiments. Significant differences between experimental groups in the quantitative RT-PCR, enzyme-linked immunosorbent assay, and luciferase assay were analyzed using Student's *t* test. Differences were considered to be significant when p was less than 0.05.

RESULTS

Down-regulation of PROS1 Expression by E_2 in HepG2-ERα Cells and Human Normal Heptocytes—Because ERα was undetectable in the original HepG2 cells by Western blotting (Fig. 1*A*), we established a HepG2-derived cell line stably expressing human ERα (HepG2-ERα) as described under "Experimental Procedures." We confirmed that the HepG2-ERα cells expressed large amounts of ERα protein equivalent to breast cancer-derived MCF7 cells as determined by Western blot analysis (Fig. 1*A*). The HepG2-ERα cells treated with E_2 showed significantly decreased levels of PS mRNA, but the original HepG2 cells and HepG2-Mock cells did not (Fig. 1*B*). Also, E_2 treatment down-regulated PS antigen significantly in the HepG2-ERα cells (Fig. 1*C*). In addition, we also demonstrated that E_2 treatment down-regulated PS mRNA by 60% in hNHep, which expressed a high level of ERα protein (Fig. 1, *D* and *E*).

Luciferase Reporter Assay—We next examined *PROS1* promoter activity by conducting a luciferase reporter assay and