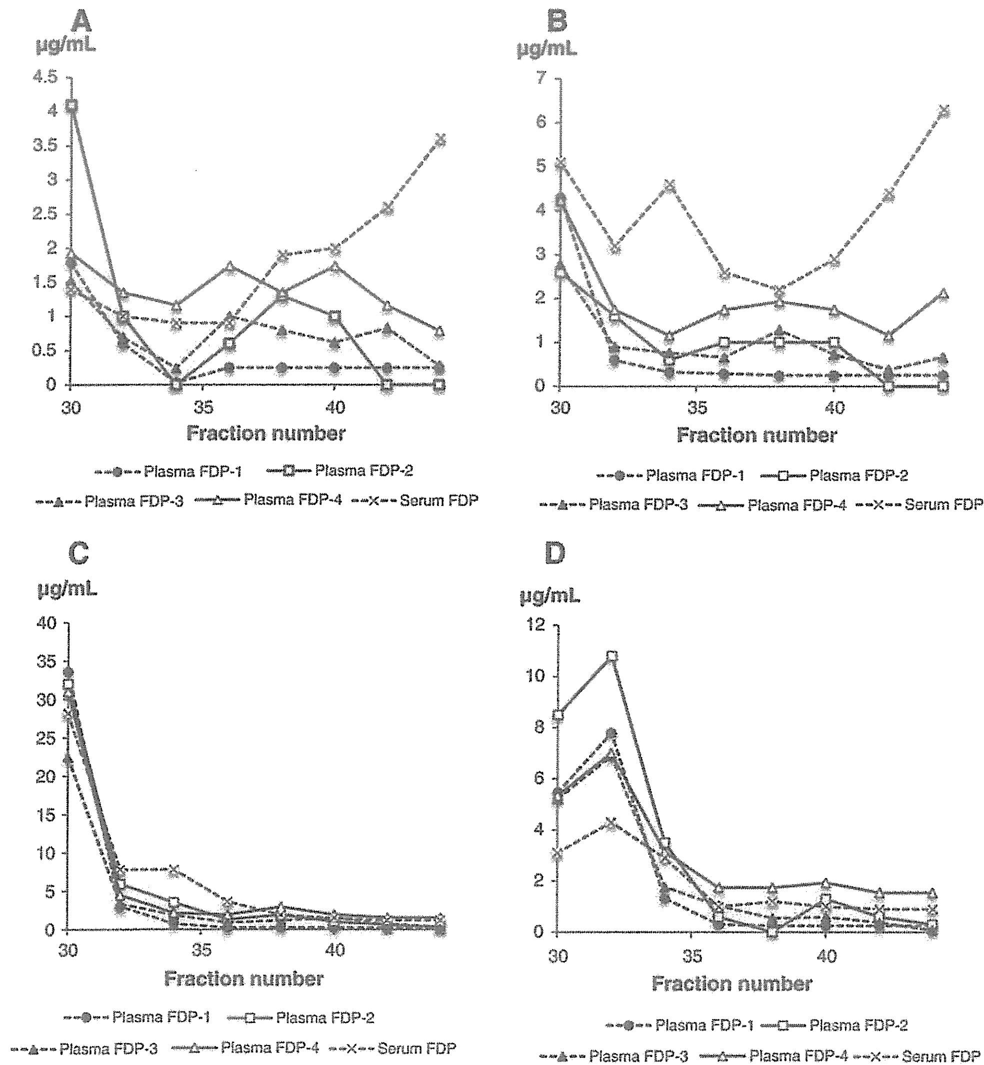

Fig. 7. Reactivity of the monoclonal antibodies used in laboratory plasma FDP reagents to the molecular variants of FDP in clinical samples. Gel filtration column chromatography fractions of clinical samples A (A), B (B), C (C), and D (D) were subjected to quantification with plasma FDP reagents. FDP concentrations of the fractions were determined with serum FDP-2 (cross), plasma FDP-1 (closed circle), plasma FDP-2 (open square), plasma FDP-3 (closed triangle), and plasma FDP-4 (open triangle).

and low serum FDP-2/D-dimer-2 ratios (C, 1.0; D, 1.0) were subjected to gel filtration column chromatography on Sephacryl S-300. The underlying diseases of the four patients were lung cancer (A), prostate cancer (B), sepsis (C), and pneumonia (D). Fractions of column chromatography were analyzed by Western blotting with the polyclonal antibody against human fibrinogen (Fig. 5), and D-dimer levels (Fig. 6) and plasma FDP levels (Fig. 7) were quantified as above. Western blot analysis with the anti-fibrinogen polyclonal antibody of the fractions (Fig. 5) revealed that the protein bands recognized by the antibody migrating approximately 130 kDa and 90 kDa (arrows) was higher in the fractions of samples A and B than that in samples C and D.

Reactivity of the monoclonal antibodies used in laboratory FDP reagents to the molecular variants of FDP in clinical samples

Various FDP forms in samples A and B (Fig. 6A, B) were quantified in fractions #30–44 with serum FDP-2. However, none of the D-dimer reagents could detect the FDP (D-dimer) well in fractions #32–44 of these samples. The FDP of samples C and D (Fig. 6C, D) was higher in fractions #30–34 than in fractions #36–44. D-dimer-1– -6 could detect the FDP of these samples in fractions 30 and 32. These results suggest that a wide variety of molecular forms of FDP were present in samples A and B (high FDP level and high serum FDP-2/D-dimer-2 ratio), and low molecular weight FDP molecular forms were not accurately quantified with D-dimer-1–6. The nature of FDP in clinical samples A and B may be different from that of *in vitro*-generated FDP. The presence of low molecular FDP forms indicated by arrows in Western blotting (Fig. 5) may partly account for this difference. These FDP forms may be FgDP or very degraded FDP missing monoclonal antibody epitopes. The results of analysis of clinical samples C and D also suggest that the high molecular forms of FDP may be the main variants of FDP molecules in samples C and D (high FDP level and low serum FDP/D-dimer ratio), and that only the high molecular FDP variants could be similarly quantified with D-dimer-1, D-dimer-2, D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6, as with serum FDP-2.

Various FDP forms in samples A and B (high FDP level and high serum FDP/D-dimer ratio) (Fig. 7A, B) were detected in fractions #30–44. Plasma FDP-2, -3, and -4 could also detect the FDP variants of

these samples in fractions #32–44. However, plasma FDP-1 failed to detect the low weight FDP forms of these samples in fractions #32–44 (Fig. 7A, B). The FDP variants in samples C and D were detected with plasma FDP-1– plasma FDP-4, similar to that with the serum FDP reagent. Though the relative values were different, plasma FDP-2–plasma FDP-4 could quantify the various FDP that formed in samples C and D in a similar manner to that with serum FDP-2. The reactivity of plasma FDP-1 to the low molecular FDP forms may be similar to D-dimer-1, D-dimer-2, D-dimer-5, and D-dimer-6.

It is possible that FgDP may be present in clinical samples A and B and may be detected by serum FDP-2 in the gel filtration column chromatography fractions. This was suggested because D-dimer-3 could not detect significant D-dimer signals in samples A and B in fractions #34–44, whereas plasma FDP-2, plasma FDP-3, and plasma FDP-4 could. Therefore, the presence of FgDP in the gel filtration column chromatography fractions of samples A and B remains to be determined.

Discussion

Various biomarkers have been developed to diagnose coagulopathy and are commercially available in clinical laboratories. Though serum FDP testing has been the standard assay used to detect thrombolysis following thrombus formation, many D-dimer reagents and plasma FDP reagents have been developed and are being widely used without standardization in clinical laboratory testing [6–8,10,11]. D-dimer reagents are considered to be specific to cross-linked fibrin derived FDP [6–8,10,11,17]. This characteristic provides the basis for the advantage of D-dimer reagents over serum FDP reagents. Plasma FDP testing enables the detection of not only cross-linked fibrin derived FDP, but also FgDP. These reagents are able to detect fibrin-derived fragments in plasma without using special test tubes for FDP. However, most of these reagents have so far not been evaluated simultaneously using the same materials and clinical samples, and have also not yet been standardized. The DIC diagnosis criteria of the Japanese Ministry of Health, Labour, and Welfare established in 1988 utilized serum FDP as a diagnostic score for DIC with cut-off values [4,18]. The DIC diagnosis criteria for acute medicine established by the Japanese Association for Acute Medicine in 2005 allows for the conversion of D-dimer values to FDP values by applying respective coefficients [1,19]. D-dimer testing has been used as a negative predictive value to rule out the presence of VTE [11–13]. Because of the importance of this test, these reagents should be standardized for the accurate diagnosis of coagulopathy such as DIC and VTE. The present study evaluated 6 D-dimer reagents and 4 plasma FDP reagents simultaneously with the same *in vitro*-generated cross-linked FDP and clinical samples to elucidate the nature of the monoclonal antibodies used in these reagents.

The results of the present study showed that the reactivity of the monoclonal antibodies used in D-dimer reagents was distinct. At least four (D-dimer-1, D-dimer-2, D-dimer-5, D-dimer-6) of six D-dimer reagents may have lower affinity for the low molecular forms of cross-linked FDP than for the very high and high molecular weight molecular forms of FDP (Table 1). This feature differs from the other two D-dimer reagents (D-dimer-3 and D-dimer-4). Three (plasma FDP-2, plasma FDP-3, and plasma FDP-4) of the four plasma FDP reagents had similar reactivity for *in vitro*-generated cross-linked FDP, while reactivity for the *in vitro*-generated FDP of plasma FDP-1 was distinct.

Analysis of clinical samples in the present study (Fig. 4) showed that the amount of low molecular weight FDP forms relative to that of the high molecular weight forms may deviate based upon the underlying disease. Analysis of the four representative clinical samples suggested that the reactivity of the D-dimer reagents for the high and low molecular weight forms of FDP was virtually consistent with that of *in vitro*-generated FDP molecules. The results of the present study also suggest that the reactivity of plasma FDP reagents for the high molecular weight and low molecular weight forms of FDP was nearly consistent with that for *in vitro*-generated FDP molecules.

In conclusion, the present study revealed that the reactivity of the monoclonal antibodies used in clinical laboratory testing to quantify FDP and D-dimer to various FDP molecular forms is distinct, and that FDP and D-dimer values in clinical samples may differ when measured with two different reagents. Although the results of our study also suggest that the development of a "universal" calibrator may be very difficult, standardization of D-dimer reagents and plasma FDP reagents is required for the accurate diagnosis of coagulopathy. One possible alternative approach would be the use of two monoclonal antibodies, with one having good affinity to very high to high molecular weight FDP variants and the other having good affinity to middle to low molecular weight FDP variants.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgments

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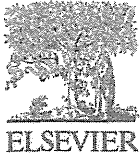

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Evaluation of hemostatic biomarker abnormalities that precede platelet count decline in critically ill patients with sepsis☆,☆☆

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Keywords:
Sepsis;
Coagulopathy;
Platelet;
Protein C;
α_2-Plasmin inhibitor;
Early diagnosis;
Critically ill

Abstract

Purpose: The hemostatic biomarkers for early diagnosis of sepsis-associated coagulopathy have not been identified. The purpose of this study was to evaluate hemostatic biomarker abnormalities preceding a decrease in platelet count, which is a surrogate indicator of overt coagulopathy in sepsis.

Materials and Methods: Seventy-five septic patients with a platelet count more than $80 \times 10^3/\mu L$ were retrospectively analyzed. Hemostatic biomarkers at intensive care unit admission were compared between patients with and patients without a subsequent decrease in platelet count (≥30% within 5 days), and the ability of biomarkers to predict a decrease in platelet count was evaluated.

Results: Forty-two patients (56.0%) developed a subsequent decrease in platelet count. Severity of illness, incidence of organ dysfunction, and 28-day mortality rate were higher in patients with a subsequent decrease in platelet count. There were significant differences between patients with and patients without a subsequent decrease in platelet count in prothrombin time–international normalized ratio, fibrinogen, thrombin-antithrombin complex, antithrombin, protein C (PC), plasminogen, and α_2-plasmin inhibitor (α_2-PI). Receiver operating characteristic curve analysis showed that PC (area under the curve, 0.869; 95% confidence interval, 0.699-0.951) and α_2-PI (area under the curve, 0.885; 95% confidence interval, 0.714-0.959) were strong predictors of a subsequent decrease in platelet count.

☆ Authors' contributions: K.K. conceived and designed the study. K.K. and S.T. prepared the data for analysis. K.K. conducted the data analysis. S.M. assisted with interpretation of the results. Y.S., J.M., and S.N. supervised the study. K.K. and S.M. drafted the article. All authors read and approved the manuscript. K.K. and S.M. take responsibility for the manuscript as a whole.

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Conclusions: Decreased PC and α_2-PI activity preceded a decrease in platelet count in intensive care unit patients with sepsis.
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1. Introduction

Coagulation and fibrinolytic abnormalities are observed in most patients with sepsis [1]. Severe inflammation in sepsis is associated with tissue factor–mediated activation of coagulation, which leads to thrombin generation and results in widespread fibrin deposition. The severity of coagulopathy in sepsis ranges from subclinical abnormalities, which are detectable by a mild increase in fibrin degradation products (FDPs) and prolongation of global clotting times, to fulminant disseminated intravascular coagulation (DIC), characterized by widespread microvascular thrombosis [2]. A number of studies have reported on the association between DIC and organ failure and found that DIC is an independent risk factor for mortality in patients with sepsis [2-5]. Early diagnosis and treatment may therefore improve outcomes in septic patients with DIC [6].

The International Society on Thrombosis and Haemostasis (ISTH) criteria are currently widely used for diagnosing DIC [7] and are a strong independent predictor of mortality in patients with severe sepsis [8]. Although the ISTH criteria for overt DIC are simple and clinically useful, they have some limitations to be applied for early stage of DIC. The ISTH criteria define nonovert DIC as the stage before overt DIC, for the purpose of early diagnosis [7]. However, previous studies have shown that few patients with nonovert DIC progress to overt DIC and that mortality rates are similar between patients with nonovert DIC and patients with overt DIC [8], suggesting that septic coagulopathy diagnosed according to the ISTH criteria for nonovert DIC may not necessarily be an early stage of overt DIC.

Previous studies have evaluated a number of hemostatic biomarkers including D-dimer, antithrombin (AT), thrombin-AT complex (TAT), plasmin–α_2-plasmin inhibitor complex (PIC), and plasminogen activator inhibitor-1 (PAI-1); however, no single marker that can effectively diagnose early stage of DIC has been identified [9,10]. It is therefore important to develop clinical markers that can detect progression of septic coagulopathy in initial phase, so that early intervention can be instituted.

The objective of this study was to evaluate the ability of hemostatic biomarkers for predicting progression of coagulopathy in septic patients admitted to the intensive care unit (ICU). We used a decrease in platelet count as a marker for overt stage of septic coagulopathy. Platelet activation, consumption, and destruction may occur at the endothelial cell surface as a result of thrombin generation and fibrin meshwork formation secondary to coagulation activation. Platelet count decreases over a few days after the development of sepsis [5], which may indicate ongoing activation of coagulation [11]. Thrombocytopenia may reflect the advanced stage of DIC, which is associated with late death in patients with severe sepsis [5,12]. We therefore considered that a decreasing platelet count could be an indicator of disease progression in sepsis-induced coagulopathy.

2. Methods

2.1. Patients

The medical records of all patients admitted to the ICU at Jichi Medical University Hospital from September 2010 to December 2011 were retrospectively reviewed. Patients with a diagnosis of sepsis and a platelet count of more than $80 \times 10^3/\mu L$ on the day of ICU admission were included in the study. Sepsis was defined as fulfillment of at least 2 of the 4 criteria for systemic inflammatory response syndrome [13] and proven or suspected infection. Exclusion criteria were as follows: age younger than 18 years, prior hematologic disorder including platelet disorder, liver cirrhosis or failure, chronic renal failure with dialysis, history of chemotherapy, anticoagulation therapy with or without AT substitution, and blood transfusion during the preceding 4 weeks. This study was approved by the Institutional Research Ethics Committee of Jichi Medical University, which did not consider informed consent to be necessary because of the study design.

Our facility provides 24-hour coverage of attending ICU physicians. Management of patients followed the Surviving Sepsis Campaign Guideline [14], with the goal of initial resuscitation and infection control. Treatment for DIC was at the discretion of the responsible ICU physicians. The basic approach to treatment was anticoagulation therapy using gabexate mesilate (a serine protease inhibitor) [15,16], with or without AT substitution therapy. Some patients with a bleeding risk, or with complications, were transfused with platelet concentrate or fresh-frozen plasma at the discretion of the treating physicians.

2.2. Data collection

Descriptive data including demographic data, diagnoses, sources of infection, and clinical data were collected from the electronic medical records of all eligible patients. Acute Physiology and Chronic Health Evaluation (APACHE) II [17] and Simplified Acute Physiology (SAPS) II [18] scores were calculated to estimate the severity of disease within the

first 24 hours of ICU admission. The Sequential Organ Failure Assessment (SOFA) scoring system [19] was used to evaluate organ dysfunction during the first 7 days of ICU stay or until ICU discharge. The ISTH criteria were used to diagnose overt and nonovert DIC, with scores calculated for each of the dates tested. Prognosis was evaluated by ICU-free days during the first 28 days [20] and all-cause 28-day mortality.

2.3. Biomarker measurements

Platelet counts were measured on the day of ICU admission (day 1) and on each of days 2 to 5. Prothrombin time–international normalized ratio (PT-INR), activated partial thromboplastin time (APTT), fibrinogen, FDP, AT, protein C (PC), plasminogen, α_2-plasmin inhibitor (α_2-PI), TAT, and PIC were measured on day 1. Assays of hemostatic parameters were performed using the CS-2100i automatic coagulation analyzer (Sysmex, Hyogo, Japan). Antithrombin, PC, plasminogen, and α_2-PI were measured using Berichrom assays (Siemens Healthcare Diagnostics, Tokyo, Japan). Thrombin-AT complex and PIC were quantitated using the TAT test F and PIC test F enzyme immunoassay, respectively (Sysmex).

2.4. Data analysis

We defined a subsequent decrease in platelet count as a decrease of 30% or more within 5 days of ICU admission. The study population was grouped according to the presence or absence of a subsequent decrease in platelet count. Differences between groups were analyzed using the Student t test for normally distributed variables and the Mann-Whitney U test for nonnormally distributed variables. Categorical data were compared using the χ^2 test or Fisher exact test.

Receiver operating characteristic (ROC) curve analysis was performed to calculate the area under the curve (AUC) for coagulation and fibrinolytic biomarkers, and the AUCs were compared to evaluate their ability to predict a subsequent decrease in platelet count. Cutoff values were calculated by maximizing the sum of sensitivity and specificity.

All P values were 2 tailed, and $P < .05$ was considered statistically significant. Data were analyzed using JMP version 10 (SAS Institute, Tokyo, Japan).

3. Results

3.1. Characteristics of the 75 eligible patients with sepsis

Of the 1343 patients admitted to the ICU during the study period, 108 had a diagnosis of sepsis on the day of ICU admission. Thirty-three patients were excluded according to the study criteria, and the remaining 75 patients were included in the study. Forty-two patients developed a subsequent decrease in platelet count within 5 days of ICU admission (Fig. 1).

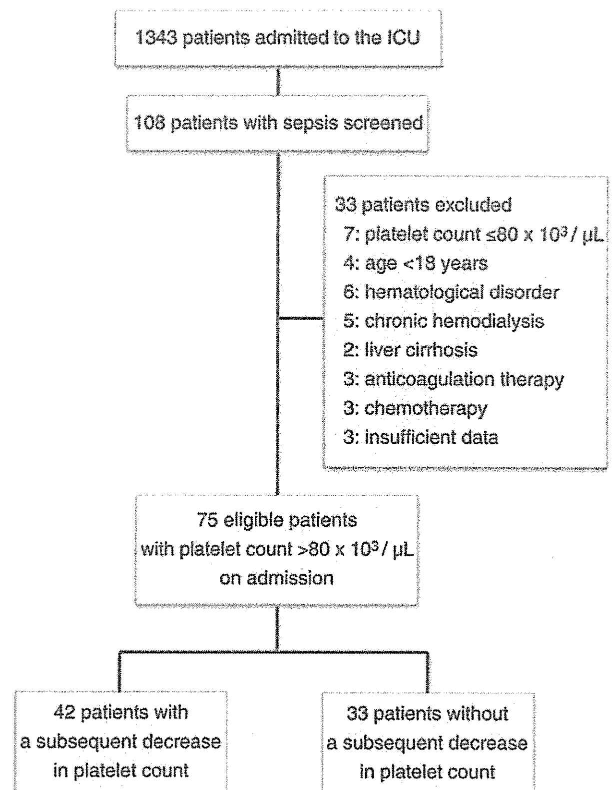

Fig. 1 Flowchart of the study patients. Sepsis was defined as fulfillment of at least 2 criteria for systemic inflammatory response syndrome and a source of infection. Eligible patients were grouped according to the presence or absence of a decrease of in platelet count 30% or more within 5 days of ICU admission.

Table 1 shows the baseline characteristics of the 75 patients. The most common cause of sepsis was abdominal infection, including 26 patients (34.7%) with lower intestinal perforation, 9 (12.0%) with acute cholangitis or cholecystitis, 5 (6.7%) with intra-abdominal abscess, and 3 (4.0%) with liver abscess (Table 1). Fifty (66.7%) of the 75 patients were surgical, and 25 (33.3%) were medical. The mean APACHE II score was 23.2 ± 7.8, and the mean SAPS II score was 49.8 ± 15.5. The all-cause 28-day mortality rate was 8.0%, with all deaths occurring during the ICU stay (Table 2).

Platelet counts over time (days 1-5) in patients with and without a subsequent decrease in platelet count are shown in Fig. 2. On the day of ICU admission, platelet counts were not significantly different between patients with and patients without a subsequent decrease in platelet count. The nadir of platelet count was on day 4 in patients with a subsequent decrease in platelet count and on day 2 in patients without a subsequent decrease in platelet count. During the first 7 days

Table 1 Baseline characteristics of the 75 patients with sepsis

	All patients (n = 75)	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P[a]
Age (y)	70.0 ± 11.7	70.1 ± 11.9	70.0 ± 11.5	.98
Male	41 (54.7)	18 (42.9)	23 (69.7)	.035
Sepsis				
Pneumonia	15 (20.0)	7 (16.7)	8 (24.2)	.56
Abdominal infection	46 (61.3)	30 (71.4)	16 (48.5)	.057
Urinary tract infection	5 (6.7)	3 (7.1)	2 (6.1)	1.00
Soft tissue infection	9 (12.0)	2 (4.8)	7 (21.2)	.038
Comorbidity				
IHD	5 (6.7)	2 (4.8)	3 (9.1)	.65
CHF	5 (6.7)	2 (4.8)	3 (9.1)	.65
COPD	4 (5.3)	2 (4.8)	2 (6.1)	1.00
CVD	2 (2.7)	0 (0.0)	2 (6.1)	.19
CKD	5 (6.7)	4 (9.5)	1 (3.0)	.38
Severity of illness				
APACHE II score	23.2 ± 7.8	25.1 ± 8.2	20.8 ± 6.8	.021
SAPS II score	49.8 ± 15.5	55.5 ± 16.1	42.9 ± 11.8	.0004
DIC score on admission				
ISTH overt	2.6 ± 1.3	2.7 ± 1.5	2.5 ± 1.1	.51
ISTH nonovert	5.0 ± 1.8	5.2 ± 1.9	4.8 ± 1.5	.33

Data are expressed as mean ± SD or number (%).
IHD indicates ischemic heart disease; CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; CKD, chronic kidney disease.
[a] Comparison of groups with and without a subsequent decrease in platelet count.

of ICU stay, circulatory failure and renal dysfunction were more frequent in patients with than without a subsequent decrease in platelet count (Table 2). Patients with a subsequent decrease in platelet count had a higher mortality rate than did patients without a subsequent decrease in platelet count (Table 2).

Table 2 Interventions, organ dysfunction, and prognosis in 75 patients with sepsis

	All patients (n = 75)	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P[a]
Transfusion (days 1-7)				
Red blood cell	18 (24.0)	11 (26.2)	7 (21.2)	.62
Platelet	9 (12.0)	9 (21.4)	0 (0.0)	.0039
Fresh-frozen plasma	16 (21.3)	15 (35.7)	1 (3.0)	.0005
Treatment for DIC (days 1-7)				
Gabexate mesilate	29 (38.7)	28 (66.7)	1 (3.0)	<.0001
Antithrombin	21 (28.0)	21 (50.0)	0 (0.0)	<.0001
Organ dysfunction (days 1-7)				
CVS (SOFA ≥ 3)	39 (52.0)	30 (71.4)	9 (27.3)	.0002
Lung (SOFA ≥ 3)	26 (48.0)	23 (54.8)	13 (39.4)	.25
Renal (SOFA ≥ 2)	16 (21.3)	14 (33.3)	2 (6.1)	.0045
Liver (SOFA ≥ 2)	28 (37.3)	18 (42.9)	10 (30.3)	.34
DIC				
ISTH overt	23 (30.7)	21 (50.0)	2 (6.1)	<.0001
ISTH nonovert	54 (72.0)	38 (90.5)	16 (48.5)	<.0001
Prognosis				
ICU-free days	19 (11.5-23)	18 (7-21)	22 (15-23.5)	.027
ICU mortality	6 (8.0)	6 (14.6)	0 (0.0)	.031
28-d mortality	6 (8.0)	6 (14.6)	0 (0.0)	.031

Data are expressed as median (interquartile range) or number (%).
CVS indicates cardiovascular system.
[a] Comparison of groups with and without a subsequent decrease in platelet count.

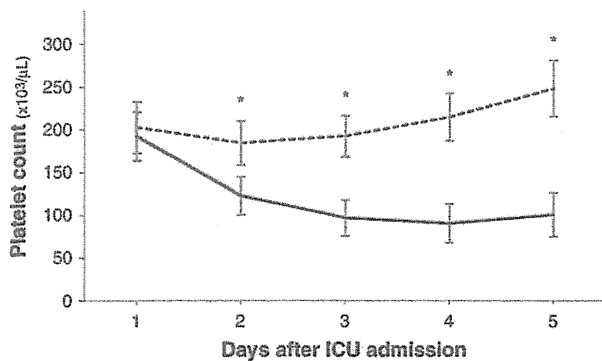

Fig. 2 Time course of platelet counts during the first 5 days of ICU stay in patients with sepsis, showing patients with a subsequent decrease in platelet count (solid line) vs patients without a subsequent decrease in platelet count (dotted line). A subsequent decrease in platelet count was defined as a decrease of 30% or more within 5 days of ICU admission. Day 1, the day of ICU admission. Data are expressed as mean and 95% CIs; $*P < .05$.

3.2. Relationships of hemostatic biomarkers with a subsequent decrease in platelet count in patients with sepsis

We evaluated coagulation and fibrinolytic markers on the day of ICU admission and examined their relationship with a subsequent decrease in platelet count. There were significant differences between patients with and patients without a subsequent decrease in platelet count in PT-INR (median, 1.33 vs 1.17; $P = .048$), fibrinogen (322 vs 526, $P = .0014$), TAT (13.8 vs 7.2, $P < .0001$), AT (51.1 vs 62.7, $P = .0099$), PC (35.1 vs 64.2, $P = .0002$), plasminogen (50.7 vs 85.3, $P = .0007$), and α_2-PI (56.3 vs 83.9, $P = .0001$) (Table 3). There were no significant differences between the 2 groups in APTT, FDP, or PIC.

3.3. Ability of hemostatic biomarkers to predict a subsequent decrease in platelet count in patients with sepsis

We conducted ROC curve analysis to evaluate the ability of hemostatic biomarkers to predict a subsequent decrease in platelet count. The AUC and best calculated cutoff values for prediction of a subsequent decrease in platelet count are shown in Table 4. The AUC for prediction of a subsequent decrease in platelet count was high for α_2-PI (0.885), PC (0.869), plasminogen (0.846), and TAT (0.846), compared with PT-INR (0.639) and fibrinogen (0.666). Interestingly, the ability to predict a subsequent decrease in platelet count was significantly higher for PC (AUC, 0.869; 95% confidence interval [CI], 0.699-0.951; $P = .0098$) and α_2-PI (AUC, 0.885; 95% CI, 0.714-0.959; $P = .029$), in pairwise comparison with PT-INR (AUC, 0.639; 95% CI, 0.432-0.799) (Fig. 3).

4. Discussion

In this retrospective study, we evaluated the usefulness of a single measurement of coagulation and fibrinolytic biomarkers on the day of ICU admission, for providing simple and prompt assessment for progression of coagulopathy in patients with sepsis. Our results show that decreased PC and α_2-PI activity were strong predictors of a subsequent decrease in platelet count, which was considered as an indicator of overt coagulopathy in sepsis.

Currently, there are no widely accepted diagnostic criteria for early stage of septic coagulopathy. Intervention to coagulopathy in initial phase might be one of the key factors in improving outcomes in patients with sepsis. Several researchers have recently established diagnostic scoring systems for the acute phase of septic coagulopathy [21-23], but these systems depend partly on changes in biomarker levels from baseline, which take at least 2 days to identify. Our results suggest that the progression of septic coagulopathy could be detected by a single measurement of PC and α_2-PI, which would help to develop early diagnostic criteria for septic coagulopathy.

Previous studies have evaluated the prognostic values of hemostatic biomarkers in patients with sepsis, but the reported results have been inconsistent [1,9,21,24]. These conflicting results may be partly caused by the multiple

Table 3 Comparison of coagulation and fibrinolytic markers on the day of ICU admission

	Reference range	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P
Platelet count ($\times 10^3/\mu L$)	130-369	181 (123-245)	197 (162-234)	.52
PT-INR	0.9-1.2	1.33 (1.14-1.53)	1.17 (1.11-1.34)	.048
APTT (s)	23.1-36.3	44.2 (32.9-59.3)	40.6 (36.1-48.3)	.71
Fibrinogen (mg/dL)	129-271	322 (212-493)	526 (380-649)	.0014
FDP (µg/mL)	0-5.0	20.4 (12.3-30.5)	16.8 (11.2-24.3)	.21
TAT (ng/mL)	<2.4	13.8 (9.9-22.1)	7.2 (5.4-9.3)	<.0001
PIC (µg/mL)	<0.9	0.9 (0.7-1.9)	1.4 (0.8-1.8)	.19
AT (%)	88-116	51.1 (36.9-62.5)	62.7 (47.8-74.1)	.0099
PC (%)	67-129	35.1 (28.1-55.8)	64.2 (56.5-79.8)	.0002
Plasminogen (%)	85-120	50.7 (40.4-68.3)	85.3 (73.9-91.9)	.0007
α_2-PI (%)	83-115	56.5 (48.3-67.6)	83.9 (75.2-94.7)	.0001

Table 4 ROC curve analyses showing AUCs for prediction of a subsequent decrease in platelet count and cutoff values maximizing the sum of sensitivity and specificity

	AUC	*P*	Cutoff value	Sensitivity	Specificity
Platelet count	0.544	.79	$157 \times 10^3/\mu L$	0.36	0.82
PT-INR	0.639	.012	1.37	0.48	0.79
APTT	0.526	.26	48.9 s	0.45	0.76
Fibrinogen	0.666	.0019	462 mg/dL	0.71	0.66
FDP	0.587	.045	29.4 μg/mL	0.33	0.88
TAT	0.846	<.0001	13 ng/mL	0.59	1.0
PIC	0.399	.57	2.7 μg/mL	0.24	0.88
AT	0.734	.0071	55.8%	0.67	0.66
PC	0.869	<.0001	41.4%	0.67	0.94
Plasminogen	0.846	.0012	72.8%	0.91	0.77
α_2-PI	0.885	<.0001	73.3%	0.92	0.75

interactive systemic factors that are involved in the pathogenesis of organ failure and the risk of mortality, although cytokine-induced coagulopathy is considered to play a major role in the process [25]. In addition, not only the development but also subsequent progression or persistence of coagulopathy are associated with poor prognosis in patients with sepsis [9,21]. We therefore focused on the progression of coagulopathy as an outcome in patients in the acute phase of sepsis.

Because there are no established criteria for assessing the progression of septic coagulopathy, we used decreasing in platelet count as a marker of disease progression. Most diagnostic criteria for DIC, including the ISTH criteria, primarily use global coagulation tests such as PT, platelet count, fibrinogen, and FDP for scoring. These markers change differently over time as coagulopathy progresses. Production of FDP may be suppressed in severe coagulopathy because of a massive increase in PAI-1 level, and the fibrinogen level tends to increase with acute-phase behavior in septic patients [26]. In addition, appropriate thresholds for the diagnosis of septic coagulopathy have not been determined. Although coagulopathy is universal in severe sepsis, only about 30% of patients in a large multicenter trial met the ISTH criteria for overt DIC [4]. The current diagnostic criteria are not useful for the evaluation of disease progression in septic patients with coagulopathy.

Thrombocytopenia is common in critically ill patients with an incidence of 40% to 50% [27], and a 30% decrease in platelet count during ICU admission has been shown to be associated with increased mortality [28]. Thrombocytopenia can be a result of many causes such as blood loss, hemodilution, thrombotic microangiopathy, and immune and drug-induced thrombocytopenia. However, septic coagulopathy is the most common underlying cause [26]. Although a diagnosis of DIC must be supported by multiple laboratory tests and other causes of thrombocytopenia should be ruled out, thrombocytopenia is a relevant marker of DIC [5]. In addition, it has been clinically recognized that thrombocytopenia may be a warning sign of severe sepsis and that the decrease in platelet count is related to the severity of sepsis [29]. In our study, a significant decrease in platelet count after ICU admission was associated with disease severity, the incidence of organ dysfunction, and the 28-day mortality rate (Tables 1 and 2).

In our study, AT, PC, plasminogen, α_2-PI activity, and TAT on the day of ICU admission had high predictive values for a subsequent decrease in platelet count in patients with sepsis. In sepsis, physiological anticoagulation mechanisms such as the AT and PC systems are impaired because of increased consumption, impaired synthesis, extravasation from vessels, and degradation by several proteolytic enzymes such as neutrophil elastase [30,31]. We found that AT and PC activity were significantly decreased in patients with a subsequent decrease in platelet count compared with patients without a subsequent decrease in platelet count, which may have been caused by increased consumption. However, mild to moderately decreased AT and PC activity were also observed in patients without a subsequent decrease in platelet count (Table 1). Asakura et al [32] reported that AT and PC activity were related to the plasma albumin level and that decreased AT and PC activity were observed in septic patients without DIC. The mechanisms of decreased AT and PC activity in patients without a subsequent decrease in platelet count may include impaired synthesis, or degradation associated with inflammation, rather than consumption coagulopathy.

We found that PC was a stronger predictor of a subsequent decrease in platelet count than AT, which is somewhat inconsistent with the results of previous studies. Several investigators have reported that AT had a higher [33] or comparable [21] association with poor prognosis, compared with PC. However, Nilsson et al [34] showed that PC had a higher correlation with the results of global clotting tests than AT. Protein C has anticoagulant and fibrinolytic properties with neutralization of PAI-1 [35], whereas AT is an anticoagulant that mainly inhibits thrombin and other procoagulant factors.

Measurement of fibrinolytic markers showed that plasminogen and α_2-PI activity were decreased in patients with a subsequent decrease in platelet count and were near the lower

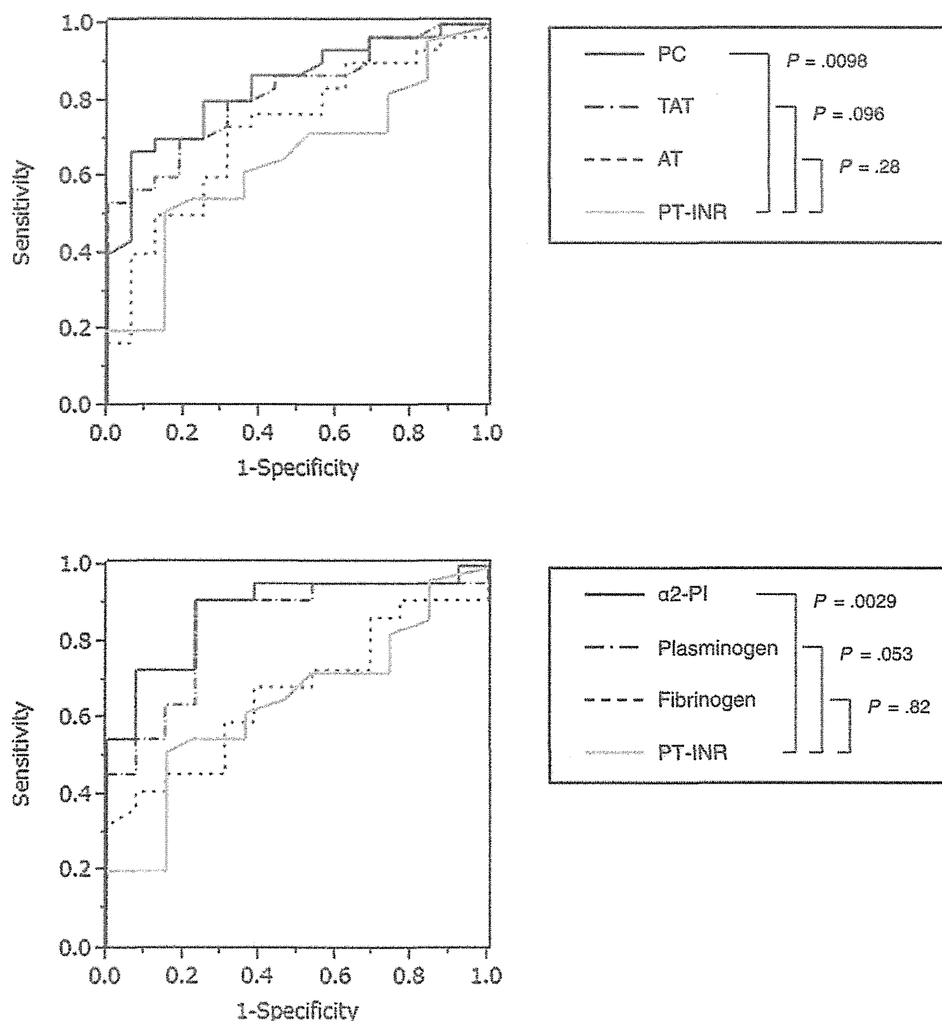

Fig. 3 Pairwise comparisons of ROC curves of biomarkers that significantly predicted a subsequent decrease in platelet count (comparison with the ROC curve of PT-INR).

limit of the reference range in patients without a subsequent decrease in platelet count. These results are consistent with those of previous study, in which only half of the 1690 patients with severe sepsis were shown to have decreased plasminogen and α_2-PI activity, although more than 80% had abnormal AT and PC activity [1]. It is known that the plasminogen activator–plasmin system is markedly depressed by PAI-1 in patients with sepsis [24]. In addition, interleukin-6 induces up-regulation of plasminogen promoter activity [36], and production of plasminogen is increased as an acute-phase protein during sepsis [37]. However, the tissue plasminogen activator level is increased in sepsis [9], and the tissue plasminogen activator/PAI-1 ratio increases with increasing severity of sepsis [33], which may explain why the levels of fibrinolytic markers were decreased under the countered increase of PAI-1.

The present study has some limitations. This was a retrospective observational study that did not promote deviation from routine clinical practices at our institution. Interventions such as treatment of DIC and blood transfusion may have influenced the levels of coagulation and fibrinolytic biomarkers and their relationships with changes in platelet count. Because our study was conducted in a single center, the relatively small size of the study population is also a limitation. A large, prospective study would be useful to validate our results.

In conclusion, decreased PC and α_2-PI activity on the day of ICU admission were strongly associated with a subsequent decrease in platelet count in patients with sepsis. A single measurement of these biomarkers may help to predict progression of septic coagulopathy and guide the decision-making process for early intervention.

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Suppression of lymph node and lung metastases of endometrial cancer by muscle-mediated expression of soluble vascular endothelial growth factor receptor-3

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Lymph node metastasis is the most important prognostic factor of endometrial cancer. However, effective therapy has not been established against lymph node metastasis. In this study, we explored the efficacy of gene therapy targeting lymph node metastasis of endometrial cancer by suppressing the action of vascular endothelial growth factor (VEGF)-C through soluble VEGF receptor-3 (sVEGFR-3) expression. For this purpose, we first conducted a model experiment by introducing sVEGFR-3 cDNA into an endometrial cancer cell line HEC1A and established HEC1A/sVEGFR-3 cell line with high sVEGFR-3 expression. The conditioned medium of HEC1A/sVEGFR-3 cells inhibited lymphatic endothelial cell growth *in vitro*, and sVEGFR-3 expression in HEC1A cells suppressed *in vivo* lymph node and lung metastases without inhibiting the growth of a subcutaneously inoculated tumor. To validate the therapeutic efficacy, adeno-associated virus vectors encoding sVEGFR-3 were injected into the skeletal muscle of mice with lymph node metastasis. Lymph node and lung metastases of HEC1A cells were completely suppressed by the muscle-mediated expression of sVEGFR-3 using adeno-associated virus vectors. These results suggest the possibility of gene therapy against lymph node and lung metastases of endometrial cancer by using muscle-mediated expression of sVEGFR-3. (*Cancer Sci*, doi: 10.1111/cas.12184, 2013)

Endometrial cancer is the most commonly encountered gynecologic malignancy and the fourth most common malignant tumor in the USA.[1] Because this cancer is often detected at an early stage while it is still confined to the uterus, the overall survival rate exceeds 80%.[1] However, the prognosis of advanced endometrial cancer remains poor.[2,3] Although various attempts have been made to treat advanced endometrial cancer, including surgery, radiotherapy and multidrug chemotherapy, satisfactory progress has not been achieved. In fact, overall treatment results in endometrial cancer have not improved over the past 30 years.[1] The most important prognostic factor in endometrial cancer is extrauterine spread, especially lymph node and lung metastases.[4] Therefore, to improve the prognosis of endometrial cancer, it is necessary to develop effective therapy against such advanced conditions.

Factors related to lymphangiogenesis and lymph node metastasis have been clarified recently. Among these factors, vascular endothelial growth factor (VEGF)-C, which is the natural ligand for VEGF receptor-3 (VEGFR-3), is one of the most important. VEGF-C binds to VEGFR-3 and induces its tyrosine autophosphorylation. VEGF-C is specific to the lymphatic vascular system and mediates lymphangiogenesis.[5] In malignant tissues, the tumor cells and stromal cells promote VEGF-C secretion, thereby inducing lymphangiogenesis and lymph node metastasis.[6] The soluble form of VEGFR-3 (sVEGFR-3) is a potent inhibitor of VEGF-C signaling, which inhibits fetal lymphangiogenesis and induces regression of already formed lymphatic vessels.[7] Therefore, lymph node metastasis of a malignant tumor may be controlled by the action of sVEGFR-3.

Recently, we developed a murine model for lymph node metastasis using orthotopic injection of an endometrial cancer cell line.[8] Based on the study, we sought to investigate the efficacy of sVEGFR3 by its constitutive expression. For this purpose, the adeno-associated virus (AAV) vector is appropriate. The AAV is a widely–used vector derived from a non-pathogenic virus, and long-term transgene expression can be obtained following intramuscular injection.[9] We have reported the efficacy of muscle-mediated soluble Flt-1 expression using AAV vectors in both subcutaneous and intraperitoneally disseminated ovarian cancer.[10] In this study, we explored the efficacy of gene therapy against metastases of endometrial cancer by muscle-mediated expression of sVEGFR-3 using AAV vectors.

Materials and Methods

Cells and plasmids. The human endometrial cancer cell line HEC1A[11] was obtained from the Japanese Collection of Research Bioresources, where the cell line is authenticated by the Multiplex-PCR method using short tandem repeats.[12] The HEC1A was cultured in DMEM/F12 (GIBCO, Grand Island, NY, USA) supplemented with 10% inactivated FCS, 100 U/mL of penicillin and 100 μg/mL of streptomycin (GIBCO) at 37°C in a 5% CO_2 atmosphere. Human neonatal dermal lymphatic endothelial cells (LEC) were purchased from Angio-Bio (Del Mar, CA, USA) and maintained in EGM-MV2 BulletKit (Cambrex, East Rutherford, NJ, USA) supplemented with 10% inactivated FCS at 37°C in a 5% CO_2 atmosphere. All cell lines were maintained for less than 3 months after resuscitation. The cDNA of sVEGFR-3 was cloned by PCR using a human lung cDNA library (Stratagene, La Jolla, CA, USA) as a template, with the primers previously described.[7] Cloned sVEGFR-3 cDNA was inserted into the multi-cloning site (MCS) of pSecTagHygroB vector (Stratagene) to generate

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a VEGFR-3-expression plasmid. A luciferase (LUC)-encoding plasmid was also constructed and used as a control vector. p2ITR-sVEGFR-3 is an sVEGFR-3 expression plasmid prepared by incorporating human sVEGFR-3 cDNA into the EcoRI site of pAAV-MCS (Stratagene).

Development of stably transduced cells. Either an sVEGFR3-expression or a LUC-expression plasmid was introduced into the HEC1A cells using the standard calcium phosphate precipitation method.[13] After transfection, the cells were cultured and selected in the presence of 200 μg/mL of hygromycin B (Invitrogen, Carlsbad, CA, USA). After 4 weeks, the hygromycin B-resistant HEC1A/sVEGFR-3 and HEC1A/LUC cell lines were established and maintained thereafter in the presence of 200 μg/mL of hygromycin B.

Adeno-associated virus vector production. Adeno-associated virus vectors were produced by triple-plasmid transfection to 293 cells (Stratagene) using p2ITR-sVEGFR-3, the helper plasmid for adenovirus genes,[14] and the helper plasmid for AAV1.[15,16] A plasmid encoding human coagulation factor IX (hfIX) gene was used to prepare the control AAV vector.[17] The vector stocks were purified using cesium chloride density-gradient ultracentrifugation, and the titer was determined by dot blot and real-time PCR, as described previously.[18] The primers were designed to amplify the cytomegalovirus promoter sequence, and the forward and reverse primers were 5′-GTA TTT ACG GTA AAC TGC CCA CTT-3′ and 5′-AGT CCC ATA AGG TCA TGT ACT GG-3′, respectively.

Vascular endothelial growth factor-C and soluble vascular endothelial growth factor receptor-3 quantitation. HEC1A, HEC1A/LUC and HEC1A/sVEGFR-3 cells were inoculated in 10-cm dishes and cultured in a 10% FCS-supplemented DMEM/F12 medium. When the cells grew to approximately 80% confluence, the culture supernatant was replaced with serum-free culture medium. After 48 h, the culture supernatant was recovered. The concentration of VEGF-C in the supernatant of HEC1A was determined using a Quantikine Human VEGF-C enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). The concentrations of sVEGFR-3 in the supernatant of HEC1A, HEC1A/LUC and HEC1A/sVEGFR3 were determined by DuoSet Human VEGFR-3 (R&D Systems).

Lymphatic endothelial cells proliferation assay. Conditioned media were generated by culturing 1×10^6 HEC1A/LUC cells or HEC1A/sVEGFR-3 cells in 2 mL of serum-free DMEM/F12 for 48 h. LEC (5×10^3/well) were plated in 96-well plates in EGM-MV2 plus 5% FCS containing 50% of either conditioned medium with 100 ng/mL VEGF-C (R&D Systems). LEC proliferation was assessed by a colorimetric assay using Cell Proliferation Kit II (XTT; Boehringer Mannheim Biochemica, Mannheim, Germany) 48 h after plating.

Tumor cell transduction model: subcutaneously inoculated tumor growth. Four to six-week-old female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) were used in the experiment. HEC1A/LUC or HEC1A/sVEGFR-3 cells (5×10^6) were subcutaneously transplanted into the backs of the mice, and tumor sizes were measured once a week using a micrometer caliper. Tumor volume was calculated using the formula: volume = (short diameter)^2 × (long diameter) × 0.5.[19]

Evaluation of metastasis. HEC1A/LUC or HEC1A/sVEGFR-3 cells (5×10^6) were injected into the uterine cavities of pentobarbital sodium-anesthetized, laparotomized mice, as described previously.[8] After 8 weeks, metastatic lesions were thoroughly investigated and counted.

Therapeutic model using adeno-associated virus vector: evaluation of metastasis in orthotopically inoculated model. HEC1A cells (5×10^6) were injected into the uterine cavities of pentobarbital sodium-anesthetized, laparotomized mice, as described previously.[8] At the same time AAV1-hfIX or AAV1-sVEGFR-3 vector (2.5×10^{12} genome copy) was injected into the hind-limb skeletal muscles of the mice. Eight weeks after injection, the metastatic changes were extensively investigated and numbers of enlarged lymph nodes and lung metastases were counted.

Statistical analysis. Intergroup differences were tested for significance using Student's *t*-test. A *P*-value <0.05 was considered significant.

Results

Detection of vascular endothelial growth factor-C and soluble vascular endothelial growth factor receptor-3 in culture supernatants. The concentration of VEGF-C in the culture supernatant of HEC1A cells was 235 ± 12 pg/mL. In the culture supernatant of HEC1A/sVEGFR-3 cells, 45.0 ± 3.2 pg/mL of sVEGFR-3 was detected, but no sVEGFR-3 was detected in the culture supernatant of either HEC1A or HEC1A/LUC cells.

Inhibitory effects of soluble vascular endothelial growth factor receptor-3 on *in vitro* lymphatic endothelial cells growth. The effect of the sVEGFR-3 expression of HEC1A/sVEGFR-3 cells on the action of VEGF-C was estimated using *in vitro* cultures of LEC. The number of LEC in EGM-MV2 culture medium, including 100 ng/mL recombinant VEGF-C plus 50% conditioned medium from HEC1A/sVEGFR-3 cells, was significantly smaller than that in the control (Fig. 1a–c, $P < 0.01$). We concluded that the mitogenic effect of VEGF-C on LEC was abrogated by the presence of sVEGFR-3 in the HEC1A/sVEGFR-3 conditioned medium.

Tumor cell transduction model: subcutaneously inoculated tumor growth. The tumor growth curves of HEC1A/LUC and HEC1A/sVEGFR-3 show no significant differences between the two groups (Fig. 2). This indicates that expression of sVEGFR-3 did not affect the growth of subcutaneously inoculated tumors.

Lymph node metastasis. The effects of sVEGFR-3 gene expression on lymph node metastasis *in vivo* are shown (Fig. 3a–c). The mean number of lymph node metastases 8 weeks after injection was 1.0 ± 0.7 in the control group, but no lymph node metastases were observed in the HEC1A/s VEGFR-3-injected group (Fig. 3c), indicating that sVEGFR-3 inhibited lymph node metastasis of the HEC1A cells.

Lung metastasis. After thorough investigation for metastasis, we noticed lung metastasis in these animals. Therefore, we focused on the number of lung metastases along with the number of lymph node metastases. The effects of sVEGFR-3 gene expression on lung metastasis *in vivo* are summarized in Figure 4. The mean number of lung metastases 8 weeks after injection was 3.8 ± 0.8 in the control group, but no lung metastases were observed in the HEC1A/sVEGFR-3-injected group, indicating that sVEGFR-3 completely inhibited lung metastasis of the HEC1A cells.

Therapeutic model using adeno-associated virus vector. The efficacy of muscle-mediated sVEGFR-3 expression was evaluated in lymph node and lung metastases models using HEC1A cells. As shown in Figure 5, the mean number of lymph node metastases 8 weeks after injection of HEC1A cells was 2.4 ± 0.5 in the control group, while no lymph node metastases were observed in the AAV1-sVEGFR-3-injected group. Moreover, the mean number of lung metastases 8 weeks after injection of HEC1A cells was 5.7 ± 2.1 in the control group, while no lung metastases were observed in the AAV1-sVEGFR-3-injected group (Fig. 6a–c). Thus, we observed a significant therapeutic effect in both lymph node and lung metastases.

Discussion

In this study, we explored the possibility of gene therapy targeted at lymph node and lung metastasis using muscle-mediated expression of sVEGFR-3 as a new treatment modality

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for advanced endometrial cancer. Our results show that sVEGFR-3 in the conditioned medium of sVEGFR-3-transduced endometrial cancer cells inhibited LEC growth *in vitro*, and sVEGFR-3 expression in endometrial cancer cells suppressed

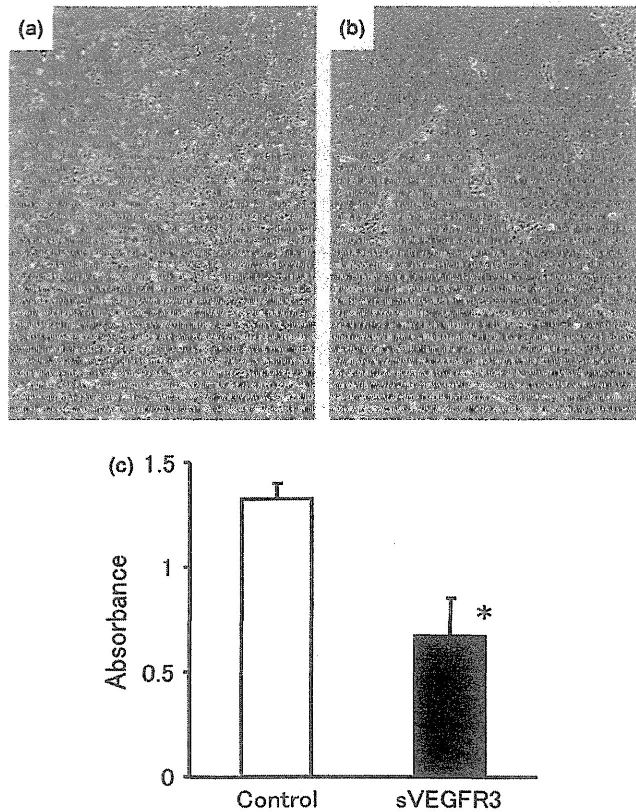


Fig. 1. Suppression of vascular endothelial growth factor (VEGF)-C-driven lymphatic endothelial cell (LEC) proliferation by conditioned medium of soluble vascular endothelial growth factor receptor-3 (sVEGFR-3)-expressing cells. Cells were plated at 5×10^3 cells/well in 96-well plates, and 50% sVEGFR-3-conditioned medium or luciferase-conditioned medium was added with 100 ng/mL recombinant human VEGF-C. The number of LEC with 50% sVEGFR-3-conditioned medium (b) was clearly smaller than that with control (a). The cells were counted by colorimetric assay 48 h after plating. Each bar represents the mean ± SD. (**P* < 0.01) (c).

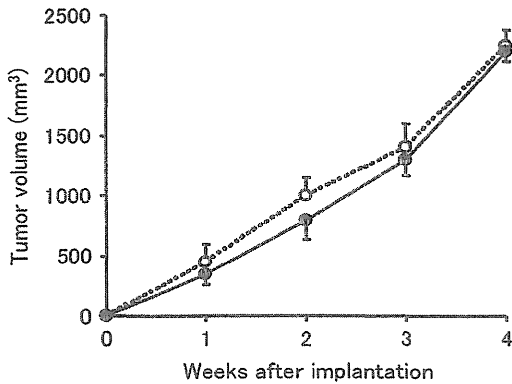
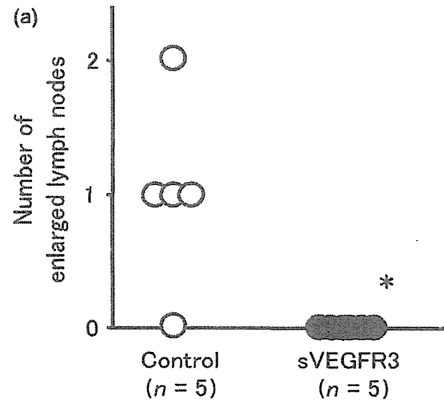


Fig. 2. The tumor growth curves of HEC1A/luciferase and HEC1A /vascular endothelial growth factor receptor-3. Tumor cells were subcutaneously injected into the backs of mice, and the sizes of tumors were measured every week. There were no significant differences between the two groups. Each bar represents the mean ± SD.



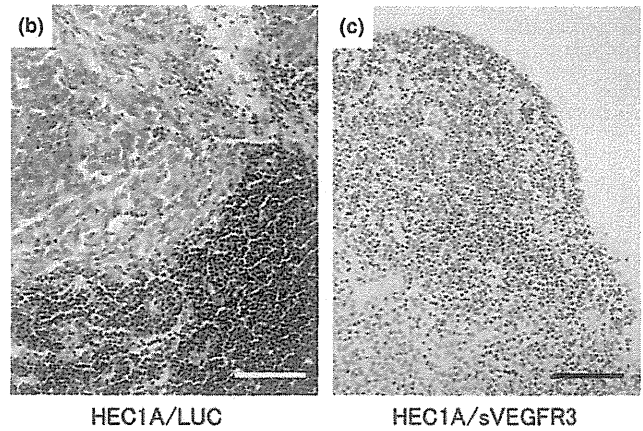


Fig. 3. (a) The number of lymph node metastases 8 weeks after injection of HEC1A/luciferase and HEC1A/vascular endothelial growth factor receptor-3 (sVEGFR-3) cells. Lymph node metastases were observed in the control group (b), while no lymph node metastases were observed in the HEC1A/sVEGFR-3-injected group (c). The mean number of lymph node metastases was 1.0 ± 0.7 in the control group, while no lymph node metastases were observed in the HEC1A/sVEGFR-3-injected group. Bars represent 100 μm.

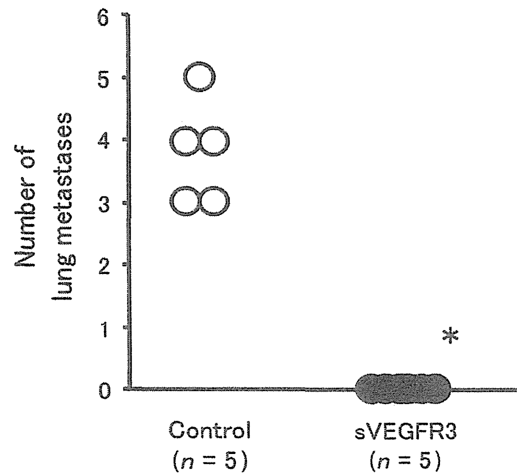


Fig. 4. The number of lung metastases 8 weeks after injection of HEC1A/luciferase and HEC1A/vascular endothelial growth factor receptor-3 (sVEGFR-3) cells. The mean number of lung metastases 8 weeks after injection was 3.8 ± 0.8 in the control group, while no lung metastases were observed in the HEC1A/sVEGFR-3-injected group.

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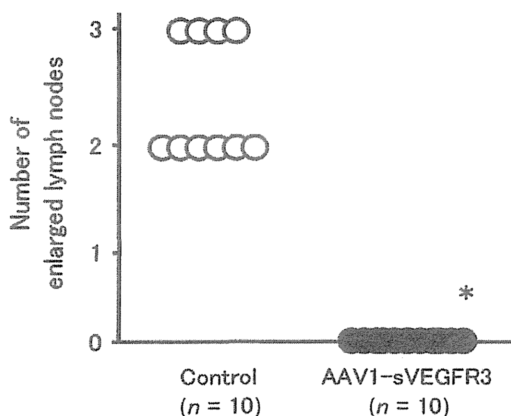
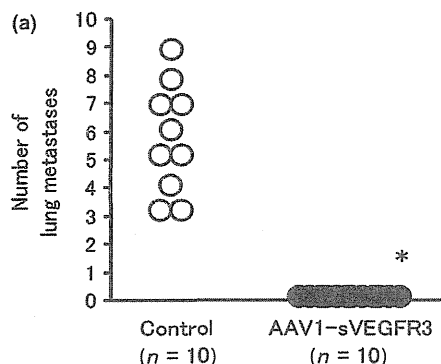


Fig. 5. The number of lymph node metastases 8 weeks after injection of HEC1A cells in the mice that had received intramuscular injections of AAV1-sVEGFR-3 or control vector. The mean number of lymph node metastases was 2.4 ± 0.5 in the control group, while no lymph node metastases were observed in the AAV1-sVEGFR-3-injected group. sVEGFR-3, vascular endothelial growth factor receptor-3.



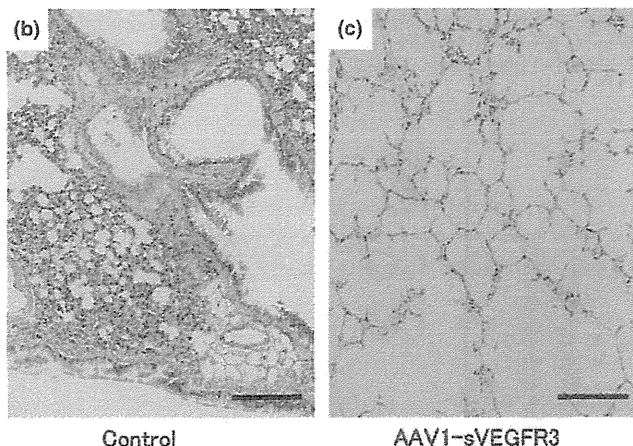


Fig. 6. Lung metastases 8 weeks after injection of HEC1A cells in the mice that had received intramuscular injections of AAV1-sVEGFR-3 or control vector. The mean number of lung metastases was 5.7 ± 2.1 in the control group, while no lung metastases were observed in the AAV1-sVEGFR-3-injected group (a). Lung metastases were observed in the control group (b), while no lung metastases were observed in the AAV1-sVEGFR-3-injected group (c). Bars represent 100 μm. sVEGFR-3, vascular endothelial growth factor receptor-3.

in vivo lymph node and lung metastases, although it did not inhibit the growth of subcutaneously inoculated tumors. In addition, lymph node and lung metastases of endometrial cancer cells were suppressed by muscle-mediated expression of sVEGFR-3 using AAV vectors.

Lymph node metastasis is the most important prognostic factor in endometrial cancer.[3,20] In this study, we tested the efficacy of a gene therapy strategy using sVEGFR-3 to suppress lymph node metastasis through VEGF-C inhibition. We first introduced sVEGFR-3 cDNA into an endometrial cancer cell line HEC1A and established a cell line (HEC1A/sVEGFR-3) with high expression of sVEGFR-3 to investigate its function. The growth of HEC1A/sVEGFR-3 did not show any difference in either *in vitro* cell proliferation (data not shown) or *in vivo* tumor expansion (Fig. 2), showing that overexpression of sVEGFR-3 did not influence the spread of endometrial cancer *per se*. In contrast, in the lymph node metastasis model using HEC1A/sVEGFR-3, lymph node metastasis was completely suppressed. Moreover, in this model, lung metastasis was also completely eliminated. Thus, we demonstrated that expression of sVEGFR-3 could control lymph node and lung metastases of endometrial cancer. Because the expression of sVEGFR-3 did not influence the growth of HEC1A cells, its action was confined to the suppression of lymph node metastasis. The VEGF-C in culture supernatant is thought to be derived from HEC1A cells, and accumulated during cell culture. In fact, we demonstrated various but similar concentrations of VEGF-C in tumor cell culture supernatant.[8] In contrast, soluble VEGFR3 was demonstrated specifically to the cells transduced by sVEGFR3 gene, as shown in the Results section.

Based on these findings, we aimed to establish a gene therapy using sVEGFR-3. For this purpose, we compared the utility of candidate vectors to attain this goal. Non-viral vectors are easier to prepare, and may be safer, but the efficacy is much weaker than viral vectors. Successful *in vivo* delivery has been limited.[21]. As for the viral vectors, many successful outcomes in clinical trials have been reported.[22] For the current study, we chose an AAV1-based vector, as it appears to be the most efficient in muscle transduction.[9,15,23] The result was that both lymph node and lung metastases of endometrial cancer cells were completely suppressed by muscle-mediated expression of sVEGFR-3. These results suggest the possibility of gene therapy targeting lymph node and lung metastases of endometrial cancer by muscle-mediated expression of sVEGFR-3. In the case of AAV vectors, a couple of weeks may be necessary for maximal transgene expression.[22,24–26] Nonetheless, as sufficient levels of expression can last over the observation period of 8 weeks, a significant outcome was obtained even when the vector was administered simultaneously to the tumor inoculation. In this study, we selected muscle tissue for gene expression. For the clinical translation, other tissues may be more appropriate: for example, liver can be efficiently targeted by AAV8 vector[26] and adipose tissue can be targeted by AAV1 vectors.[24]

This study aimed to suppress actions of VEGF-C through the expression of a soluble form of its receptor, sVEGFR3. Therefore, lymph node metastasis was suppressed as a result of lymphangiogenesis inhibition. However, because this treatment did not suppress primary tumor growth, it may be necessary to combine it with other treatment modalities such as surgery and chemotherapy in clinical practice. One recent study utilized chemotherapeutic reagents in addition to gene therapy using soluble VEGF receptors for prolonged survival in mice.[27]

Recently, we developed a lymph node metastasis model using orthotopic injection of endometrial cancer.[8] In that report, only lymph node metastasis was noted. During the current series of experiments, metastatic foci of the lungs could barely be recognized from the surface, and we noticed lung metastasis after extensive microscopic examination.

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Therefore, the number of metastasis was counted based on the microscopic examination of the tissue sections. As for lung metastasis, both lymphatic and hematogenous routes are known. It is not easy to determine the route of metastasis solely by histopathological examination. Nonetheless, we assume that in human endometrial cancer, the main route of lung metastasis is lymphatic as lymph node metastasis is detected in more than half of the patients with lung metastasis.[28] Also, in the present study, lung metastasis of endometrial cancer was completely suppressed as a result of controlling lymph node metastasis with sVEGFR-3.

In this study, we demonstrated the efficacy of sVEGFR-3 at one vector dose. As both lymph node and lung metastasis were completely eliminated, there is a possibility that the therapeutic efficacy can be demonstrated at lower vector doses. In addition, in our observation, no side effects were noted in the mice, including in behavior, body weight and muscle tissue. However, for application to human therapy, these points need to be clarified in more detail.

Few studies concerning molecular-targeted therapy or gene therapy for endometrial cancer have been reported. Gene therapy against lymph node and lung metastases, which we propose here, could be beneficial for patients with advanced endometrial cancer. New therapeutic modalities, including this one, are expected to result in improved outcomes for endometrial cancer.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest to declare.

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Prophylaxis and Treatment of Alzheimer's Disease by Delivery of an Adeno-Associated Virus Encoding a Monoclonal Antibody Targeting the Amyloid Beta Protein

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Abstract

We previously reported on a monoclonal antibody (mAb) that targeted amyloid beta (Aß) protein. Repeated injection of that mAb reduced the accumulation of Aß protein in the brain of human Aß transgenic mice (Tg2576). In the present study, cDNA encoding the heavy and light chains of this mAb were subcloned into an adeno-associated virus type 1 (AAV) vector with a 2A/furin adapter. A single intramuscular injection of 3.0×10^{10} viral genome of these AAV vectors into C57BL/6 mice generated serum anti-Aß Ab levels up to 0.3 mg/ml. Anti-Aß Ab levels in excess of 0.1 mg/ml were maintained for up to 64 weeks. The effect of AAV administration on Aß levels in vivo was examined. A significant decrease in Aß levels in the brain of Tg2576 mice treated at 5 months (prophylactic) or 10 months (therapeutic) of age was observed. These results support the use of AAV vector encoding anti-Aß Ab for the prevention and treatment of Alzheimer's disease.

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Introduction

Alzheimer's disease (AD) is a disorder characterized by a diffuse loss of neurons and the accumulation of amyloid beta (Aß) protein, followed by the production of tau protein or senile plaques in the brain [1–2]. Active immunization with Aß peptide was found to reduce the amyloid burden and improve cognitive behavior in murine AD models [3–4].

Clinical trials involving peptide immunization were suspended owing to the development of meningioencephalitis in some volunteers vaccinated with Aß peptide [5–6]. Clinical studies and autopsy results indicated aseptic meningoencephalitis, presumably induced by the T-cell responses [6–8]. Of note, several of the samples obtained from vaccinated patients demonstrated a remarkable reduction in Aß protein levels and senile plaque formation [9–10]. These results suggest that if the adverse side effects of such therapy could be avoided, immune mediated elimination of Aß protein could represent a promising therapy for AD.

Based on these observations, the efficacy of intravenous delivery of humanized monoclonal antibodies (mAbs) against Aß was examined [11–13]. Despite the widespread reduction in Aß plaques, the passive transfer of mAb reduced AD-like symptoms in only a subset of patients [10]. This observation suggests that neuronal degeneration may occur during the early stages of AD, before the appearance of large Aß aggregates. Thus, it is important to eliminate Aß oligomers at the earliest stages of AD. Previously, we developed a mAb targeting the Aß1–13 peptide. Prophylactic delivery of this mAb or its F(ab')2 fragments to human Aß transgenic mice (Tg2576) effectively prevented the accumulation of Aß protein and plaques [14]. However, Pfeifer et al. [15] reported that anti-Aß mAb treatment could also lead to microhemorrhages in APP23 mice. Moreover, repeated high-dose mAb injections are likely to be very expensive [5,8].

A potentially safer and more efficacious strategy would be to inject an adeno-associated virus (AAV) that leads to the continuous production of anti-Aß mAb over an extended period. AAV is a nonpathogenic and poorly immunogenic virus. When used as a vector, it can transfer a gene of interest to non-dividing mammalian cells resulting in persistent transgene expression [16].

This work examines the feasibility of using an AAV vector type 1 (AAV vector) modified to encode the anti-Aß Ab to prevent or treat AD in mice. This approach avoids the need to repeatedly administer high doses of mAb. Results suggest that therapy with an Aß mAb-expressing AAV vector greatly reduce Aß accumulation in AD model mice.

Results

Production of Ab by cells transfected with the Aß mAb – expressing AAV vector

We first determined whether the transduction of the new Aß mAb – expressing AAV vector resulted in the production of mAb by HEK293 cells. As shown in Figure 1, we detected Abs in the cell lysates and culture supernatant of the transduced cells. Heavy (H) and Light (L) chains of the appropriate molecular weight were detected. In addition, we detected intact Ab under non – reducing condition. These results indicate that Aß mAb – expressing AAV vector-transduced cells produce proteins with the molecular weight of Abs.

Binding activity of the Ab produced by AAV vector – transduced cells

We next assessed whether the HEK293 – derived Abs could bind to monomeric Aß protein and oligomerized Aß protein similar to those found in the brain of patients with AD [17]. Results show that culture supernatant derived from Aß mAb-expressing AAV vector-transduced HEK293 cells bound to monomers, dimers, trimers, and tetramers of Aß protein (Figure 2A).

We then analyzed whether Aß mAb – expressing vector-produced Abs bound to Aß aggregates by observing sliced brain sections from Tg2576 mice. Aß aggregates were clearly detected in the brain sections using a polyclonal antibody against Aß1 – 42, anti – Aß1–13 mAb (IIA2), and the culture supernatant from Aß mAb – expressing vector – transduced HEK293 cells (Figure 2B). These results suggest that functional anti-Aß mAb is produced by cells transduced with this Aß mAb – expressing vector.

Inhibition of hippocampal cell death by Aß aggregates using culture supernatant from AAV-transduced cells

It is hypothesized that early AD is characterized by the aggregation of Aß protein, and is followed by abnormal tau phosphorylation leading to massive neuronal cell death in the brain. We therefore examined whether the culture supernatant from Aß mAb - expressing AAV vector-transduced cells could inhibit the death of primary hippocampal cells. As shown in Figure 3, synthetic soluble Aß aggregates killed hippocampal cells. This cell death was significantly reduced by the addition of culture supernatant from Aß mAb - expressing vector-transduced cells at 6 h and 24 h after incubation. These results suggest that the culture supernatant of AAV-transduced cells can inhibit the death of primary hippocampal cells.

Antibody titers of mice infected with the Aß mAb-expressing AAV vector

Ten weeks old C57BL/6 mice were intramuscularly (i.m.) injected with 3.0×10^{9}, 3.0×10^{10} or 3.0×10^{11} viral genome (vg) of the Aß mAb - expressing vector or 3.0×10^{11} vg of LacZ-expressing vector. After administration, serum from these mice was collected monthly and antibody titers were assayed for 64 weeks (Figure 4). Anti-Aß Ab titers peaked approximately 4 weeks after administration and then slowly declined, remaining detectable through 64 weeks of follow up. Ab titers were dose-dependent, with the greatest amount of Ab being present in mice treated with 10^{11} vg of the Aß mAb-expressing AAV vector. In contrast, no Aß-specific Abs were detected in mice injected with the LacZ-expressing AAV vector (data not shown). By 64-week post administration, 0.1 mg/ml of Aβ-specific Ab can be detected in the mice administrated with 3.0×10^{10} vg of the Aß mAb-expressing AAV vector. Considering the safe dosage range of 2.1×10^{12}–6.9×10^{13} vg/individual [18] and 2×10^{11}–1.8×10^{12} vg/kg [19] in phase 1 clinical trials of intramuscular injection of the recombinant AAV vector, we use the dose of 3.0×10^{10} vg/mouse for further in vivo study, based on the body weight ratio of human beings (60 kg) vs mouse (20 g) and the Ab titer after administration of Aβ mAb-expressing AAV vector (Figure 4).

Effect of Aß mAb-expressing AAV vector prophylaxis on Tg2576 mice

To determine whether the Aß mAb-expressing AAV vector was able to prevent Tg2576 mice from developing AD, 5-month old animals were injected once with 3.0×10^{10} vg of this vector. Whereas no Aβ protein accumulated in normal mice, there was a statistically significant increase in the amount of Aβ1-40 and Aβ1-42 present in the brain of Tg2576 animals treated with the control LacZ-expressing vector by 10 months of age. The accumulation of this protein continued to rise over time (Figure 5B). By comparison, the amount of Aβ protein present

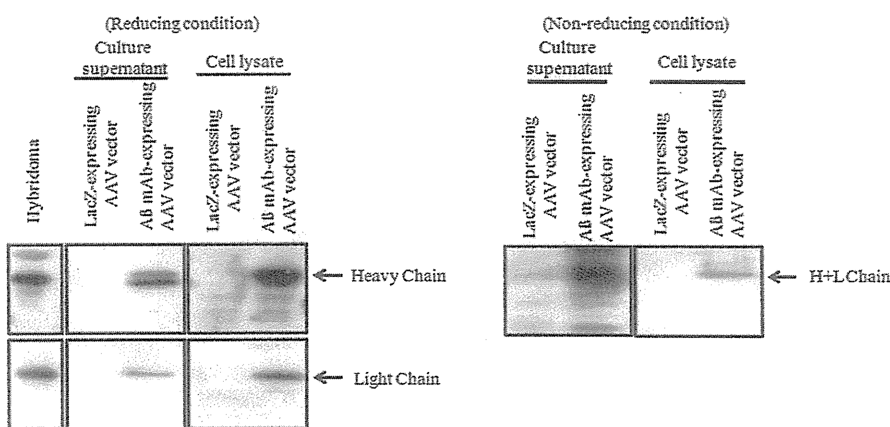

Figure 1. In vitro expression of anti - Aß Abs following the transduction of HEK293 cells with the Aß mAb – expressing AAV vector. Western blots of culture supernatant and cell lysates identify the Ig light and heavy chain (under reducing conditions) and whole Ab (under non-reducing conditions). Cells transfected with a LacZ encoding AAV vector served as negative controls.
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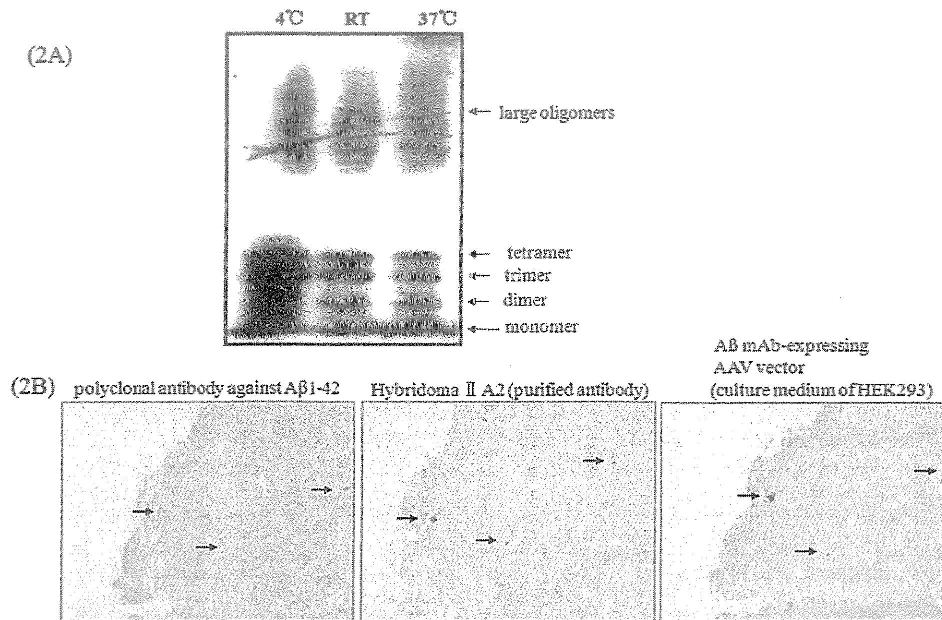

Figure 2. Binding activity of the Ab produced by Aß mAb – expressing AAV vector-transduced cells. A) Binding of Ab from Aß mAb – expressing AAV vector transduced HEK293 cells to synthetic Aß peptides, monomers and oligomers by Western blot. The Ab1-42 peptide was aggregated at 4°C, room temperature (R.T.), or 37°C as described in Materials and Methods and detected by culture supernatant derived from Aß mAb – expressing AAV vector transduced HEK293 cells. B) Anti – Aß Ab derived from transduced HEK293 bound to Aß plaques in 16-month old Tg2576 mice. The specificity of this binding was confirmed by use of polyclonal and monoclonal Abs (see details in Materials and Methods).
doi:10.1371/journal.pone.0057606.g002

in Tg2576 mice treated with the Aβ mAb-expressing AAV vector was significantly and persistently reduced (Figure 5B).

Serial sagital sections were prepared from the brains of these animals. Aß protein deposits were then visualized immunohistochemically in these sections. Both the size and number of Aβ protein containing deposits increased over time in Tg mice treated with the LacZ-expressing vector. The number of such plaques was significantly reduced among mice treated with the Aß mAb-expressing vector (Figure 5C).

Effect of Aß mAb-expressing AAV vector treatment on Tg2576 mice

We finally sought to determine whether the Aß mAb-expressing AAV vector could be used therapeutically. Ten-month old animals were injected with 3.0×10^{10} vg of vector. Aß protein continued to accumulate at 13 months in mice treated with either the LacZ or Aß mAb-expressing AAV vector. However by 15 months (and continuing through 17 months) the size and number of Aß protein containing deposits in the brains of animals treated with the Aß mAb-expressing vector was significantly reduced when compared to LacZ controls (Figure 6B). This divergence was confirmed

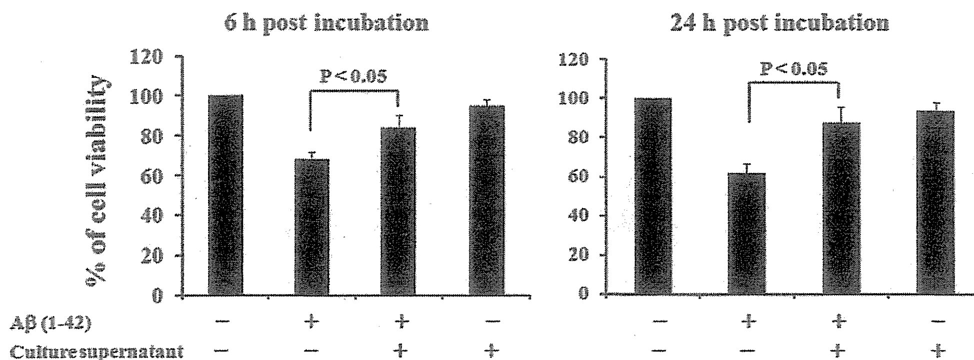

Figure 3. In vitro cytotoxicity inhibition test using primary culture hippocampal cells. The viability of primary hippocampal cells after 6–24 h of culture with 10 uM aggregated Aß protein was examined. The effect of adding culture supernatant from Aß mAb - expressing AAV vector transduced cells was also examined by MTT assay. Data represent the results of 5–8 independently analyzed samples and are presented as mean ± SE.
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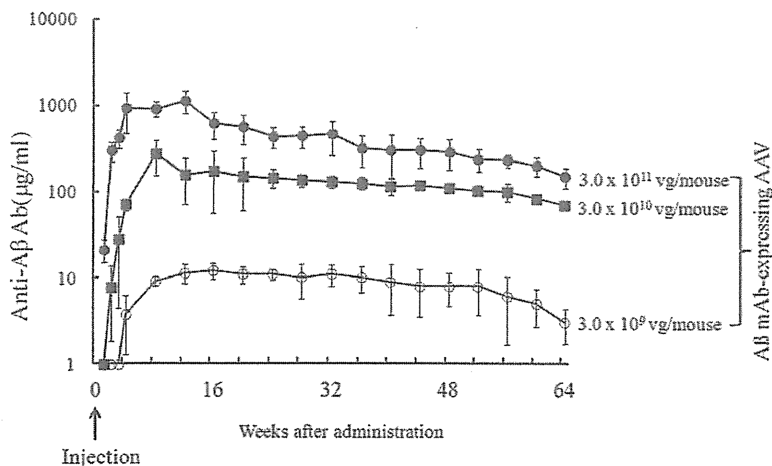

Figure 4. Kinetics of anti-Aß Ab production by mice injected with Aß mAb-expressing AAV. Normal C57BL/6 mice (n = 10/group) received a single intramuscular injection with 3.0×10^9, 3.0×10^{10}, 3.0×10^{11} vg of Aß mAb-expressing AAV or 3.0×10^{11} vg of LacZ-expressing AAV vector. The titer of IgG1 Ab binding to Aß1–42 was detected at indicated time points. Significant difference among groups received 3×10^9, 3×10^{12} and 3×10^{11} vg of Aß mAb-expressing AAV was observed from 2-week to 64-week after administration ($P<0.05$). Data are presented as mean ± SE.
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during the immunohistologic analysis of brain tissue from these animals. Aß protein containing deposits accumulated over time in the LacZ but not the Aß mAb - expressing AAV vector (Figure 6C).

Discussion

Efforts to treatment AD patients with anti-Aß Abs or through Aß peptide vaccination provided novel insights concerning the pathogenesis of AD and opened new approaches to disease therapy. In an effort to overcome limitations of earlier strategies, the current work examined the effect of delivering an Aß mAb - expressing AAV vector to Tg2576 mice (a murine model of AD). Prophylactic treatment of young (5 months) and therapeutic treatment of older (10 months) animals resulted in a significant and prolonged decrease in the amount of Aß protein accumulating in the brain (Figure 5 and 6).

The AAV vector encoded an anti-Aß mAb that bound to synthetic Aß peptides and to senile plaques present in the brains of Tg2576 mice (Figure 1 and 2). Of interest, a single 3.0×10^{10} vg dose of the Aß mAb-expressing AAV vector resulted in the production of Ab that persisted through the 64-week experimental period (Figure 4). As repeated injection of free anti-Aß mAb can have negative consequences [15], the continuous production of Ab by cells transfected in vivo may provide an ideal method for AD prophylaxis and treatment.

Previous studies investigated the utility of AAV vector for the molecular therapy of Alzheimer's disease. Those vectors encoding antigen, Ab or other factors of potential therapeutic value were examined in various animal models [20–31]. These included studies of AAV vector expressing a single-chain variable fragment (scFv) antibody against Aβ protein for AD therapy [22–26]. Those studies showed that the scFv fragment had a much shorter serum half-life than whole Ab (7–14 hrs *vs* 20 days) [32], such that the scFv fragment was more suited for intracranial delivery rather than systemic delivery [22–26]. Delivery of scFv - expressing AAV vector intracranially reduced/prevented the formation of Aβ brain plaques and improved cognitive function in AD mice [22–26], while the delivery method is likely to raise safety issues.

This study examined the effect of treating 5- or 10-month old Tg2576 mice with the Aß mAb - expressing AAV vector. Of importance, a significant decline in the concentration of Aß was found in the brains of both groups of recipient mice (monitored by ELISA and immunohistochemistry). The level of decline was similar in both groups despite the difference in when treatment was initiated (Figure 5 and 6). This may reflect the level of Aß protein being so low in young mice that the effect of therapy cannot be detected until the animals reach 15 months of age. By that time the vector had been active in both treatment groups for a sufficient period to significantly reduce the accumulation of Aß protein

Clinical trials showed that vaccination of AD patients with an Aß peptide reduced the deposition of Aß in some individuals [10]. Unfortunately, this treatment also led to the development of cerebroencephalitis in some patients, a side effect so severe that further development of this type of therapy was abandoned [11,14,33]. An alternative approach involved the intravenous administration of Abs against Aß peptide [5,8]. While effective at reducing the accumulation of Aß aggregates, the injection of anti-Aß Ab resulted in a high incidence of cerebral microhemorrhages [15]. Approximately 30% of AD patients have cerebral amyloid angiopathy (CAA)-associated microhemorrhages [34]. Shroeter et al. [35] reported that anti-Aß Ab treatment of 12-month old AD model mice resulted in a dose-dependent reduction of the occurrence of CAA after treatment. In older model mice, CAA is known to be relatively abundant [36–37]; however, the injection of a small amount of anti-Aß mAb did not increase the incidence of microhemorrhages [36–37], indicating that long-lasting expression of anti-Aß mAb by AAV vector may have some advantages in reducing the incidence of microhemorrhages. Many reports have explored the T-cell response after virus or non-virus-based vaccine against Alzheimer's. The T-cell response is very low or undetectable [38–40]. In this study, we have not explored the T-cell response, because our AAV vector expresses anti-Aβ-antibody, but not an antigen. Furthermore, we histologically evaluated the appearance of microhemorrhages and inflammation of brain in the mice treated with the Aß mAb-expressing AAV vector. No evidence of this adverse side effect was observed even in 17-month old Tg2576 mice (data not shown).