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Serum Neutrophil Extracellular Trap Levels Predict Thrombotic Microangiopathy after Allogeneic Stem Cell Transplantation

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Transplantation-associated thrombotic microangiopathy (TA-TMA) is a devastating complication of hematopoietic stem cell transplantation. TA-TMA likely represents the final stage of vascular endothelial injury; however, its pathophysiology is largely unknown, making clinical management difficult. Recently, the association of neutrophil extracellular traps (NETs) with the development of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome has been reported. Thus, we explored whether NETs are also relevant to the occurrence of TA-TMA. We retrospectively analyzed post-transplant trends of serum NET levels in 90 patients, 11 of whom developed TA-TMA. Relative to baseline (before the conditioning regimen), elevated serum NET levels either at 4 weeks after transplantation or as early as the day of transplantation were associated with significantly increased risk of TA-TMA. In contrast, thrombomodulin, a potential marker for TA-TMA, was not helpful to predict the occurrence of TA-TMA in our study. In addition, we directly detected glomerular deposition of NETs in 2 TA-TMA patients. Increased NET levels are a significant risk factor for TA-TMA, suggesting that NET level is a useful biomarker for TA-TMA.

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INTRODUCTION

Thrombotic microangiopathies (TMAs) are microvascular occlusive disorders characterized by thrombocytopenia, systemic or intrarenal aggregation of platelets, mechanical injury to erythrocytes, and ischemic organ damage [1]. The most common TMAs are thrombotic thrombocytopenic purpura (TTP), due to decreased activity of a disintegrin and metalloprotease with a thrombospondin type 1 motif domain 13 (or ADAMTS13) [2], or hemolytic uremic syndrome (HUS) [3], due to infection with Shiga toxin–producing *Escherichia coli* in diarrhea-positive HUS. However, TMAs are also associated with other clinical conditions, including allogeneic stem cell transplantation (allo-SCT), tumor, chemotherapy, pregnancy, and autoimmune disease. Although allo-SCT is highly effective for eradicating hematological malignancies, it has a higher risk of treatment-related mortality.

One devastating complication of allo-SCT is transplantation-associated TMA (TA-TMA), affecting 10% to 25% of patients [4], 60% to 75% of whom may die within 3 months [1,5]. TA-TMA is associated with a variety of risk factors, such as acute graft-versus-host disease (GVHD), infections, unrelated or HLA-mismatched donor grafts, female sex, and the use of a calcineurin inhibitor or sirolimus [1,4]. Thus, TA-TMA is a syndrome representing a "final common pathway" of vascular endothelial injury damaging the kidney and other organs after allo-SCT [1], being distinct from TTP and HUS. The effective management of TA-TMA is currently hindered by the lack of early diagnostic biomarkers for TA-TMA, and the discovery of early markers is an important step for the successful treatment of TA-TMA.

Neutrophil extracellular traps (NETs), originally described as a component of innate antimicrobial immunity, are extracellular fibrillar matrices composed of chromatin and granule proteins released by activated neutrophils [6]. NETs are known to contribute to autoimmunity and thrombosis; systemic lupus erythematosus [7,8], small-vessel vasculitis [9], pre-eclampsia [10], sepsis [11,12], transfusion-related acute lung injury [13,14], deep vein thrombosis [15], and atherosclerosis [16]. NETs in the peripheral circulation are associated with the development of several types of TMA, such as TTP and HUS [17]. The authors in the study speculated that NETs may provide a second hit that precipitates acute disease in patients at high risk for TMA [17]. To date, however, the involvement of NETs in the pathogenesis of TA-TMA remains to be elucidated. Here, we analyzed the serum NET profiles after allo-SCT to determine the effects of NETs on TA-TMA and to evaluate the use of NET levels as a predictive marker for TA-TMA.

METHODS

Patient Characteristics and Allo-SCT Procedures

We retrospectively reviewed the clinical history of patients who underwent allo-SCT in our department between September 2007 and April 2012 and survived at least 28 days after allo-SCT. Ninety patients were included in the study. Patient characteristics and allo-SCT procedures are summarized in Table 1.

Underlying diagnoses included acute myelogenous leukemia, myelodysplastic syndrome, acute lymphoblastic leukemia, malignant lymphoma,

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Table 1
Characteristics of All SCT Patients

Variables	All SCT Patients (N = 90)	TMA Patients (N = 11)
Sex, male/female	50/40	8/3
Age, y, median (range)	48.5 (17-66)	44 (21-60)
Over/under 50	40/50	5/6
Disease, AML/MDS/ALL/ML/ATL/MM/AA	34/10/16/21/7/1/1	6/1/1/3/0/0/0
SCT risk, high/standard	48/42	9/2
Disease status, progressive/controlled	36/54	8/4
Prior allo-SCT, yes/no	10/80	2/9
Donor source, R-BM/R-PB/UR-BM/CBT	17/3/43/27	1/0/6/4
ABO mismatch, major + minor/major/minor/none	13/18/19/40	0/3/1/7
Conditioning, MAC/RIC	39/51	7/4
GVHD prophylaxis		
CyA/FK506 based	16/74	1/10
Methotrexate/mycophenolate mofetil/both	47/7/18	4/1/4
Follow-up, day, median (range)	700 (98-1660)	778 (140-1051)

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; ATL, adult T cell leukemia; MM, multiple myeloma; AA, aplastic anemia; R-BM, related bone marrow; R-PB, related peripheral blood stem cell; UR-BM, unrelated bone marrow; CBT, cord blood transplantation; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; CyA, cyclosporine A.

adult T cell leukemia, multiple myeloma, and aplastic anemia. Patients were divided into high-risk and standard-risk groups, according to the transplantation risk. Standard-risk criteria were as follows: (1) acute leukemia in first complete remission phase, or (2) de novo refractory anemia and ringed sideroblasts, or (3) malignant lymphoma/multiple myeloma in complete or partial remission phase, or (4) all nonmalignant hematological diseases. All other patients were considered high risk.

Disease status at SCT was divided into controlled and progressive disease. Controlled disease was defined according to the relevant criteria: (1) partial or complete remission (acute myelogenous leukemia, acute lymphoblastic leukemia, malignant lymphoma, adult T cell leukemia, and multiple myeloma), (2) <10% marrow blasts (myelodysplastic syndrome), or (3) all nonmalignant hematological diseases. As for conditioning regimens, definitions of myeloablative conditioning and reduced-intensity conditioning were consistent with those of the reduced-intensity conditioning regimen workshop [18].

TA-TMA Diagnosis and Therapy

TA-TMA was diagnosed according to Blood and Marrow Transplant Clinical Trials Network Toxicity Committee criteria [5]: (1) erythrocyte fragmentation and ≥2 schistocytes per high-power field of a peripheral smear, (2) concurrent increased serum lactate dehydrogenase levels above institutional baseline, (3) concurrent renal dysfunction (doubling of serum creatinine levels from baseline or 50% decrease in creatinine clearance from baseline) and/or neurological dysfunction without other explanations, and (4) negative direct and indirect Coombs test results. TA-TMA severity was assessed as follows: grade 1, schistocytes without clinical consequences; grade 2, presence of schistocytes and elevated creatinine levels ≤3-fold the upper limit of normal; grade 3, presence of schistocytes and elevated creatinine levels >3-fold the upper limit of normal but not requiring dialysis; and grade 4, presence of schistocytes with renal failure either requiring dialysis and/or associated with encephalopathy. Patients who were grades 2 to 4 at least once were diagnosed with TA-TMA and included in the TMA group. Therapeutic strategies for TA-TMA, such as reduction of calcineurin inhibitor, infusion of fresh frozen plasma, administration of recombinant thrombomodulin, or plasma exchange, were determined by the attending physicians.

Serum NET Level Evaluation

Serum samples were collected at 3 different times (before conditioning regimen [PRE], day of transplantation [Day0], and in the 4th week [4WK]) and preserved at −80°C. As control samples, sera from healthy volunteers were obtained (N = 11; 9 men and 2 women; median age 25.5 years [range, 24 to 44]). Protocols were approved by the Ethics Committee of Kyoto University, and written informed consent was obtained from each patient. Serum NETs were quantified using Quant-iT PicoGreen double-stranded DNA (dsDNA) Reagent (#P7581, Molecular Probes, Eugene, OR) according to the manufacturer's instructions (PicoGreen assay). Fluorescence was recorded in a fluorometer (Fluoroskan Ascent, #5210470, Thermo Fisher Scientific K.K., Yokohama, Japan) with filter settings of 485 nm (excitation) and 538 nm (emission). Capture ELISA was used to measure the dsDNA combined with myeloperoxidase (MPO) granules (MPO-DNA ELISA) [13]. Values are expressed as absorbance above control and compared with values obtained using the PicoGreen assay.

Serum Thrombomodulin Level Evaluation

Serum thrombomodulin levels were determined using ELISA for thrombomodulin (#DTHBD0, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Immunofluorescence Staining of Kidneys Obtained from TA-TMA Patients

Paraffin-embedded kidney samples from 2 TA-TMA patients were prepared, mounted on glass slides, deparaffinized in xylene (#244-00081, Wako Pure Chemical Industries, Tokyo, Japan), and rehydrated through a graded alcohol series and distilled water. After antigen retrieval with citrate buffer (#S1700, Dako, Tokyo, Japan), specimens were blocked for 1 hour in 10% FCS in PBS (#16030-074, Molecular Probes). Primary antibody incubation was overnight at 4°C with 10 μg/mL rabbit monoclonal anti-human MPO antibody (#ab45977, Abcam, Tokyo, Japan), followed by 1 hour of incubation with anti-rabbit IgG-Alexa Fluor 488 (2 μg/mL, #A-11008, Molecular Probes). Nuclear and extracellular dsDNA were detected by staining with 500 nM Sytox Orange Nucleic Acid Stain (#S11368, Molecular Probes). Slides were coverslipped with mounting media (ProLong Gold Antifade Reagent, #P36930, Molecular Probes) and analyzed by confocal laser scanning fluorescence microscopy (Digital Eclipse C1; Nikon, Tokyo, Japan).

Statistical Analysis

Overall survival after allo-SCT was calculated with Kaplan-Meier methods and compared by log-rank tests. Nonrelapse mortality was analyzed using Gray's methods, considering relapse as a competing risk. Serum NET and thrombomodulin levels were compared by unpaired *t*-tests and repeated-measures analysis of variance with Dunnett's multiple comparisons test. Univariate analysis of the cumulative incidence of TA-TMA was performed using Gray's methods, considering early death as a competing risk; factors with significance or borderline significance ($P < .1$) were subjected to a multivariate analysis using Fine-Gray proportional hazards models. Cumulative incidence of acute GVHD (grades II to IV) and early bacterial infection (documented bacteremia up to Day 100) was calculated using Gray's method, considering relapse or early death as a competing risk. Statistical analyses were performed using R (version 2.13.0; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (version 6.00; GraphPad Software, La Jolla, CA). The alpha level of all tests or the *P* value was set at .05.

RESULTS

Incidence and Clinical Outcomes of TA-TMA

Within the median follow-up of 700 days (range, 98 to 1660 days), TA-TMA was diagnosed in 11 cases, at a median of 42 days (range, 19 to 78 days) after allo-SCT, and the cumulative incidence at Day 100 was 12.2% (95% confidence interval, 6.5% to 19.9%) (Table 2). Therapeutic interventions for TA-TMA (fresh frozen plasma infusion in 5 patients, plasma exchange in 3, and recombinant thrombomodulin administration in 1) had limited effects, resulting in the deterioration and subsequent death in 5 patients. The 1-year overall survival was significantly lower (31.2% versus 69.8% [$P < .01$]) and nonrelapse mortality significantly higher (39.0% versus 9.3% [$P = .03$]) in, respectively, the TMA group than in the non-TMA group (Figure 1A,B).

PicoGreen Assay Is Feasible for Evaluating Serum NETs

Estimations of NET levels obtained by MPO-DNA ELISA or the cell-free dsDNA PicoGreen assay were positively correlated in the analysis of 40 randomly selected specimens (Pearson's correlation coefficient, $R^2 = .57$, $P < .01$, data not shown), suggesting that NETs were the major source of the serum dsDNA detected by the PicoGreen assay in accord with

Table 2
Characteristics of Patients with TA-TMA

	Age Sex	Disease Status	Donor Source	Conditioning		GVHD Prophylaxis	Acute GVHD	TA-TMA	Encephalopathy	TA-TMA Therapy	TA-TMA Remission	Relapse	Outcome (Post-SCT Day)	
1	59F	MDS relapse	CBT	RIC	Flu/Mel/TBI	FK	III Day 43	Gr2 Day 42			22 d	N	Alive	1051
2	31F	AML relapse	R-BM	RIC	Flu/Mel	FK/MTX/MMF	I Day 59	Gr2 Day 40			49 d	Y Day 482	Death (relapse)	798
3	44M	ALL CR1	CBT	MAC	CY/TBI	FK/MMF	II Day 33	Gr3 Day 61		FFP/rTM	40 d	N	Alive	140
4	53M	AML PD	UR-BM	MAC	BU/CY	FK/MTX		Gr3 Day 20			67 d	Y Day 147	Alive	778
5	38F	ML relapse	CBT	MAC	Flu/Mel/TBI	CyA/MTX		Gr3 Day 46		FFP	N	N	Death (MOF)	52
6	54M	AML PD	CBT	MAC	CY/TBI	FK		Gr4 Day 28	Y	FFP/PE	99 d	Y Day 117	Death (relapse)	143
7	60M	AML CR1	UR-BM	RIC	Flu/Mel	FK/MTX/MMF	II Day 13	Gr4 Day 19	Y	PE	19 d	Y Day 124	Death (relapse)	263
8	60M	ML NC	UR-BM	RIC	Flu/Mel	FK/MTX/MMF	IV Day 32	Gr4 Day 55	Y	FFP	N	Y Day 33	Death (relapse)	89
9	39M	AML relapse	UR-BM	MAC	CY/TBI	FK/MTX	IV Day 23	Gr4 Day 56	Y	FFP	N	N	Death (TA-TMA)	101
10	36M	ML relapse	UR-BM	MAC	CY/TBI	FK/MTX	IV Day 54	Gr4 Day 78		HD	N	N	Death (infection)	143
11	21M	AML CR3	UR-BM	MAC	BU/CY	FK/MTX/MMF	III Day 24	Gr4 Day 31	Y	PE/HD	N	N	Death (TA-TMA)	46

CR indicates complete remission; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; PD, progressive disease; NC, no change; Flu, fludarabine; Mel, melphalan; TBI, total body irradiation; CY, cyclophosphamide; BU, busulfan; MTX, methotrexate; MMF, mycophenolate mofetil; FFP, fresh frozen plasma; ML, malignant lymphoma; rTM, recombinant thrombomodulin; PE, plasma exchange; Gr, grade; HD, hemodialysis; MOF, multiple organ failure; R-BM, related bone marrow; R-PB, related peripheral blood stem cell; UR-BM, unrelated bone marrow; CBT, cord blood transplantation; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning.

previous studies [7,11,12,14,17,19,20]. Furthermore, the Pico-Green assay proved to be a powerful time- and labor-saving tool for evaluating serum NETs in a clinical setting. NET levels in serum samples were not significantly different from those in plasma samples (data not shown).

Serum NET Levels Increase after Allo-SCT, Especially in TA-TMA Patients

We compared serum NET levels for all allo-SCT patients at PRE, Day0, and 4WK. The serum NET levels in subjects before the conditioning regimen (102.7 ± 4.9 ng/mL [mean ± standard error mean]) and in healthy control subjects (N = 10, 107.0 ± 5.2 ng/mL) were similar in both the groups (P = .77). NET levels between Day0 and before transplantation (PRE) were significant in the TMA group (108.1 ± 16.3 ng/mL versus 91.3 ± 11.7 ng/mL, P = .02) but not significant in the non-TMA group (101.8 ± 4.51 ng/mL versus 104.3 ± 5.36 ng/mL, P = .52). In contrast, the 4WK levels were significantly elevated compared with the levels before conditioning regimen in both groups (150.3 ± 13.3 ng/mL, P < .01, in the TMA group; 119.4 ± 6.2 ng/mL, P = .02, in the non-TMA group) (Figure 2A). Furthermore, the 4WK levels in the TMA group were significantly higher than those in the non-TMA group (P = .04) (Figure 2A). Next, we calculated the ratio of serum NET levels at Day0 and 4WK to those before transplant. The ratio of 4WK-to-PRE levels was significantly higher in the TMA group than in the non-TMA group (1.76 ± .13 versus 1.23 ± .06 [P < .001]) (Figure 2B). Interestingly, the ratio of Day0-to-PRE levels was also significantly higher in the TMA group (1.18 ± .05 versus 1.03 ± .02 [P = .03]) (Figure 2B).

We next examined serum levels of soluble thrombomodulin, a marker for endothelial injury, because TA-TMA is closely associated with vascular endothelial injury. The 4WK levels of thrombomodulin were significantly elevated compared with PRE levels in both the TMA and non-TMA group (Figure 2C). However, there were no differences in absolute thrombomodulin levels at 4WK (Figure 2C), Day0/PRE ratio, or 4WK/PRE ratio (Figure 2D) between the TMA and non-TMA groups, in sharp contrast to the NET levels.

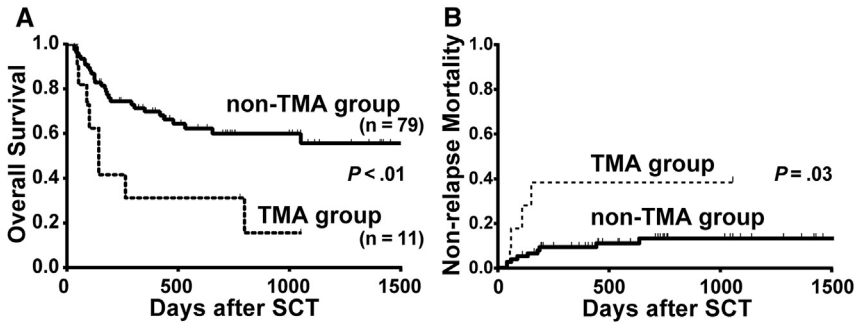

Figure 1. Survival of patients after allo-SCT. (A) Overall survival was calculated using the Kaplan-Meier method, and TMA and non-TMA groups were compared using the log-rank test. The 1-year overall survival was significantly lower (31.2% versus 69.8% [P < .01]) in the TMA group. (B) Nonrelapse mortality at 1- year was significantly higher (39.0% versus 9.3% [P = .03]) in the TMA group.

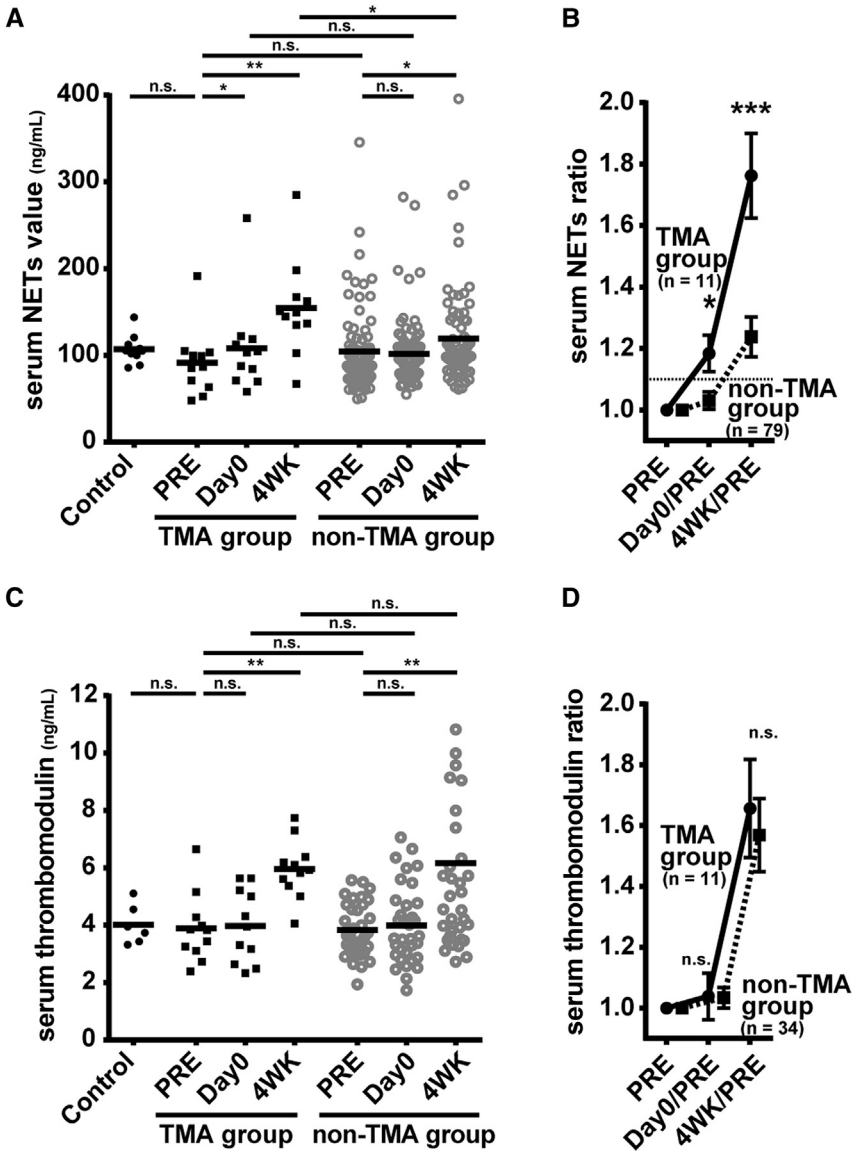

Figure 2. Serum NET trends after allo-SCT. (A) The serum NET levels before conditioning regimen (PRE), on the day of SCT (Day0), and 4 weeks after SCT (4WK) in the TMA (N = 11) and non-TMA (N = 79) groups. (B) The ratio of serum NET levels at Day0 and 4WK to PRE (Day0/PRE and 4WK/PRE) in the TMA (N = 11) and non-TMA (N = 79) groups. In the TMA group, Day0/PRE and 4WK/PRE are significantly higher than in the non-TMA group. (C) The serum thrombomodulin levels before conditioning regimen (PRE), on the day of SCT (Day0), and 4 weeks after SCT (4WK) in the TMA (N = 11) and non-TMA (N = 34) groups. (D) The ratio of serum thrombomodulin levels at Day0 and 4WK to PRE (Day0/PRE and 4WK/PRE) in the TMA (N = 11) and non-TMA (N = 34) groups. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

These results suggest that serum NET levels may be more useful as a predictive marker for TA-TMA.

NET Level Elevation Is a Significant Risk Factor for TA-TMA

To evaluate the potential of NETs as a predictive marker for TA-TMA, we created receiver operating characteristic curves of Day0/PRE and 4WK/PRE ratios (data not shown). Cut-off values (1.1 and 1.5 for Day0/PRE and 4WK/PRE, respectively) were defined to maximize the combination of sensitivity and specificity, and that of absolute NET levels at 4WK was determined just above the maximum value of healthy control subjects (140 ng/mL). Univariate analysis found a significantly higher incidence of TA-TMA in patients with either Day0/PRE >1.1 (36 cases) or 4WK/PRE >1.5 (19 cases) than in patients with either Day0/PRE ≤1.1 (54 cases; 22.2% versus 5.6%, $P = .01$) or 4WK/PRE ≤1.5 (69 cases; 31.6% versus 4.3%, $P < .01$), respectively (Figure 3A,B). Furthermore, it was noteworthy that TA-TMA was more common in patients whose absolute NET levels were >140 ng/mL 4 weeks after transplant (24 cases), compared with those with ≤140 ng/mL at the same time point (64 cases; 25.0% versus 4.7%, $P < .01$; Figure 3C). In the 4WK analysis, 2 patients who developed TA-TMA before 4 weeks after transplant were excluded.

In contrast, the incidence of TA-TMA was not related to absolute NET levels at Day0 (data not shown). Day0/PRE ratio, 4WK/PRE ratio, or absolute NET levels at 4WK were not associated with other allo-SCT–related complications, such as acute GVHD or bacterial infections (up to Day 100) (data not shown).

Other significant risk factors for TA-TMA were high-risk SCT (18.8% versus 4.7%, $P = .04$), and progressive disease (22.2% versus 5.6%, $P = .01$; Table 3). Nonsignificant risk factors included sex, older age, underlying disease, previous

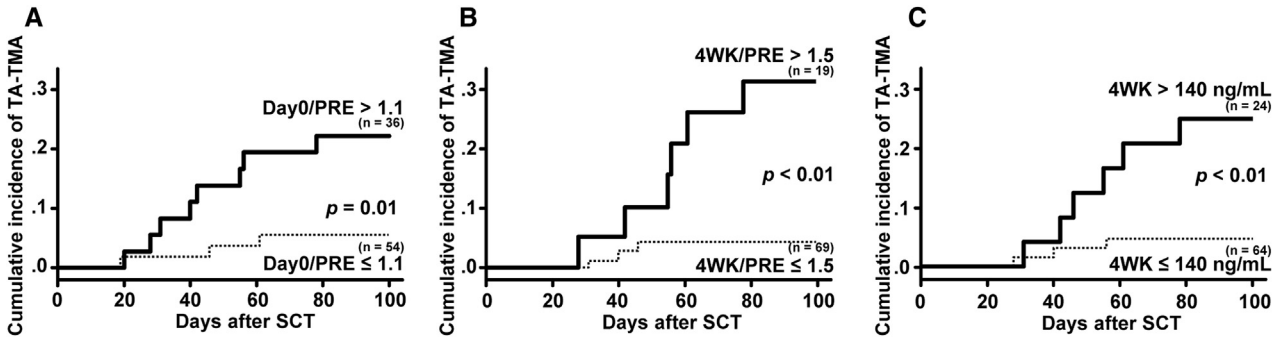

Figure 3. High NET levels are a risk factor for TA-TMA. (A) The incidence of TA-TMA relative to the Day0/PRE serum NET ratios. (B) The incidence of TA-TMA relative to the 4WK/PRE serum NET ratios. (C) The incidence of TA-TMA relative to absolute serum NET levels at 4WK. Note that elevations of either the serum NET ratios (Day0/PRE and 4WK/PRE) or absolute NET levels at 4WK are significant risk factors for TA-TMA.

allo-SCT history, donor source, blood type mismatch, conditioning regimens, type of GVHD prophylaxis, and serum thrombomodulin levels before SCT or at 4 weeks. Multivariate analysis showed that 4WK NET levels >140 ng/mL (hazard ratio 4.46; 95% confidence interval, 1.32 to 15.0; $P = .01$) and Day0/PRE ratios >1.1 (hazard ratio 3.55; 95% confidence interval, 1.03 to 12.2; $P = .04$) were significant, indicating their potential utility as biomarkers for TA-TMA (Table 3).

Identification of NET Deposition in the Glomeruli of TA-TMA Patients by Immunostaining

Kidney specimens were obtained at autopsy from patients with TA-TMA (n = 2, Cases 1 and 2). Fragmented erythrocytes, thrombosis, and fibrins were visible in the glomerulus after staining with either H & E (Figure 4A), phosphotungstic acid hematoxylin (Figure 4B), or periodic acid-Schiff (Figure 4C). After immunofluorescence staining with MPO and dsDNA, clumps of NETs were visualized intravenously as weblike, granular structures (Figure 4F,G), indicating the involvement of NET deposition in the development of TA-TMA.

DISCUSSION

Our study results demonstrated that serum NET levels were elevated 4 weeks after SCT, compared with the pretransplantation level, and this increase was especially marked in patients who developed TA-TMA. Furthermore, the ratio of either Day0/PRE or 4WK/PRE NET levels was significantly higher in the TMA group. NET deposition visualized directly in the renal glomeruli in TA-TMA patients.

At present, it is not clear whether NET formation is a cause or a consequence of TA-TMA. To solve this issue, therapeutic intervention to prevent NET formation in a prospective study is necessary. However, taking all data into consideration, we speculate that NET formation may be relevant to the pathogenesis of TA-TMA. Vascular endothelial cell injury may occur initially because of a variety of allo-SCT–related procedures, such as chemotherapy, total body irradiation, calcineurin inhibitor, and antimicrobial agents [21]. Subsequently, injured endothelium induces NET formation, and vice versa [22], resulting in a positive-feedback cycle between NET formation and endothelial injury. Indeed, NET formation occurs in proportion to the severity of endothelial damage after allo-SCT. Binding of NETs to the endothelium, especially via histones, elastase, and MPO components, is responsible for NET-mediated cytotoxicity [20,23]. NETs promote thrombus formation through interactions with platelets and erythrocytes [24]. In addition, vascular endothelial cell damage directly activates the coagulation cascade, leading to intravascular thrombosis [4]. Finally, NETs cooperate with injured endothelium to

Table 3
Risk Factors for TA-TMA

Variables	Univariate Analysis		Multivariate Analysis	
	Cumulative Incidence at 1 Year (%)	*P*	Hazard Ratio	*P*
Sex, male/female	16.0/7.5	.23		
Age, over/under 50 y	12.5/12.0	.89		
Disease, AML/MDS/ALL/ML	17.6/10.0/6.2/14.3	.81		
SCT risk, high/standard	18.8/4.7	.04*	1.62 (.18-13.9)	.66
Disease status, progressive/controlled	22.2/5.6	.01*	2.75 (.42-17.8)	.29
Prior allo-SCT, yes/no	20.0/11.2	.41		
Donor source, R-BM/R-PB/UR-BM/CBT	5.9/.0/14.0/14.8	.73		
ABO mismatch, yes/no	8.0/17.5	.17		
Conditioning, MAC/RIC	17.9/7.8	.19		
GVHD prophylaxis, CyA/FK506 based	6.2/13.5	.42		
4WK-NET value, over/under 140 ng/mL	28.0/6.2	<.01*	4.46 (1.32-15.0)	.01*
NETs Day0/PRE ratio, over/under 1.1	22.2/5.6	.01*	3.55 (1.03-12.2)	.04*
4WK-thrombomodulin level, over/under mean (6.0 ng/mL)	35.3/17.9	.19		
PRE-thrombomodulin level, over/under mean (4.0 ng/mL)	23.5/25.0	.86		

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; R-BM, related bone marrow; R-PB, related peripheral blood stem cell; UR-BM, unrelated bone marrow; CBT, cord blood transplantation; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; CyA, cyclosporine A.

* Statistically significant.

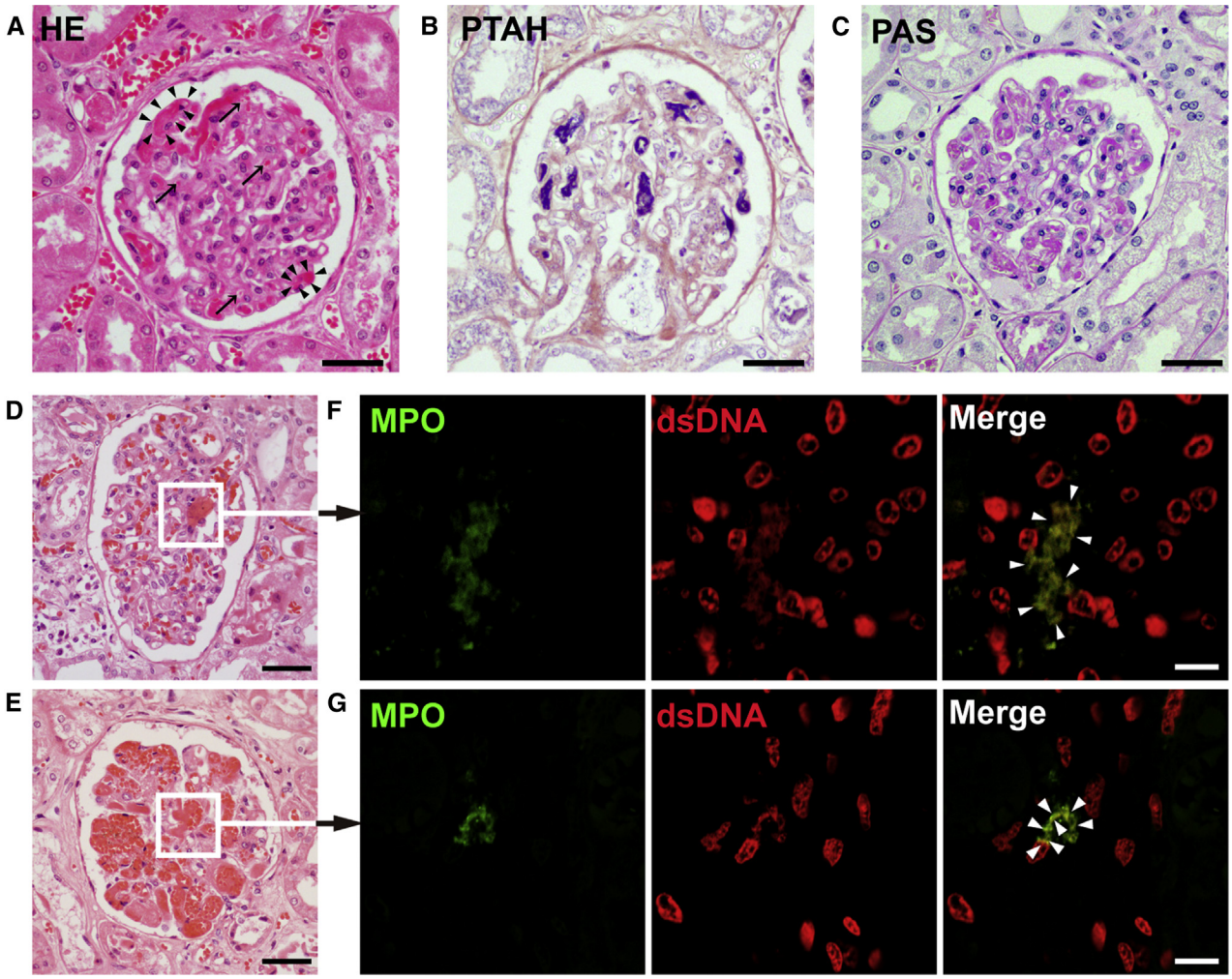

Figure 4. Immunofluorescence staining of glomeruli in patients with TA-TMA. Kidney specimens were obtained at autopsy from patients with TA-TMA. (A) H & E staining; characteristic features of TA-TMA, such as fragmented erythrocytes (arrows), thrombosis, and fibrins (arrow heads) are visible. (B) Phosphotungstic acid hematoxylin staining and (C) periodic acid-Schiff staining showing fibrin deposition in glomeruli. (D and E) H & E staining of glomeruli in Cases 1 (D) and 2 (E). (F and G) Immunofluorescence staining with MPO and dsDNA in Cases 1 (F) and 2 (G). NETs are visible as weblike structures (arrow heads). The photographs in (F) and (G) are high-power views of the boxed areas in (D) and (E), respectively. Scale bars represent 50 μm (A-E) and 10 μm (F, G).

promote thrombosis in small vessels, leading to hemolysis, thrombocytopenia, poor end-organ perfusion, and, ultimately, TA-TMA.

TA-TMA is one of the most serious complications after allo-SCT [4]. In our study, the remission rate of TA-TMA was approximately 55% (6 of 11 patients). Currently, there is no optimal management strategy for TA-TMA, and existing treatments, such as plasma exchange or administration of fresh frozen plasma and recombinant thrombomodulin, are only partially effective [21]. Early diagnosis and successful therapeutic interventions depend on a wealth of knowledge of the pathophysiology of TA-TMA and the characterization of early diagnostic biomarkers; our results show that the Day0/PRE serum NET ratio may be useful in this respect. We found a significant association between a >10% increase in serum NETs at Day0 versus PRE and TA-TMA morbidity. The increased Day0/PRE ratio may be related to endothelial cell damage after the conditioning regimens, because we did not find a direct correlation between Day0/PRE ratio and early complications, such as acute GVHD and bacterial infection.

Serum NET levels were significantly elevated at 4 weeks after SCT compared with PRE in the TMA as well as the non-TMA patients. The elevation at 4 weeks in the non-TMA group may, at least in part, reflect neutrophil activation to form NETs by microbial infections after allo-SCT [6,25,26]. NET-dependent microbe trapping usually occurs at a focal infectious site, and the presence of fragmented NETs in sera is related to the severity of infection [11,12]. It is notable that the serum NET levels at 4 weeks after SCT in the TMA group were significantly higher than in the non-TMA group. We further discovered that patients with NET levels of >140 ng/mL at 4 weeks after SCT, which was the highest value in healthy individuals, were prone to TA-TMA but were not at high risk for bacterial infections. Thus, we speculate that a positive-feedback loop between endothelial injury and NETs rather than infection may be the primary cause of elevated NETs in the TMA group. Only 2 of 11 patients in our study developed TMA earlier than 4 weeks after SCT, suggesting that the absolute NET levels at 4 week after SCT could also be a noninvasive diagnostic marker for TA-TMA, avoiding the need for invasive procedures such as biopsies of the gastrointestinal tract or kidney.

In our study, serum thrombomodulin levels in the TA-TMA group were not significantly different from those in

the non-TMA group. These results indicate that NETs may be a superior biomarker for TA-TMA. Because our current study has some limitations, such as small number and heterogeneous group of patients analyzed, a prospective study in larger populations is necessary to validate the usefulness of serum NET levels as a predictive marker for TA-TMA. Finally, disruption of circulating dsDNAs from NETs could be a potential target for treatment of TA-TMA, as suggested from animal studies of deep venous thrombosis that show DNase1 or heparin treatment suppresses thrombosis through reduced NET formation [24,27].

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