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Associations between endothelial cell activation and acute GVHD after allogeneic hematopoietic stem cell transplantation

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ABSTRACT

Hematopoietic stem cell transplantation (HSCT) involves the potential for certain serious transplant-related complications such as graft-versus-host disease (GVHD), and recovery from such complications is vital for a successful HSCT outcome. Acute GVHD (aGVHD) occurs in the early period after transplantation and is initiated by alloreactive donor T cells. The mechanisms whereby immune responses trigger this post-transplantation condition remain unclear, but endothelial cell function might play a role in this. We investigated the expression of endothelial cell activation markers such as sE-selectin, sVCAM-1, PAI-1 and microparticle (MP) in patients undergoing allogeneic HSCT. Additionally, we studied the effects of recombinant soluble thrombomodulin (rTM) on the expression of these markers. Our study cohort included 312 patients who underwent allo HSCT at 25 institutions in Japan. In the 143 patients who developed aGVHD, levels of endothelial cell activation markers were significantly higher compared to patients who did not develop aGVHD. Moreover, patients who received rTM exhibited a significantly lower frequency of aGVHD and reduced levels of endothelial cell activation markers. Our findings suggest that endothelial cell activation might be linked to aGVHD, and that rTM might at least in part act to prevent aGVHD by its effect on endothelial cells.

KEYWORDS: rTM, TAC, stem cell transplantation, sE-selectin, HMGB1

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) often involves serious transplant-related complications including graft-versus-host disease (GVHD), veno-occlusive disease (VOD), pulmonary vasculopathy, thrombotic microangiopathy (TMA) and capillary leak syndrome [1, 2]. Although the complex pathophysiology of acute (a) GVHD involves the conditioning regimen, cytokines, nitric oxide and

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non-T effector cells, cytolytic activity of donor T-cells is essential for its development [3]. The cytolytic activity of cytotoxic T-lymphocytes (CTL) is primarily mediated through effector mechanisms such as the Fas/Fas ligand (FasL) and perforin/granzyme pathways [4, 5]. Some studies have indicated that the Fas/FasL system is involved in the pathogenesis of aGVHD [3, 6]. Although the diagnosis of vascular complications in patients undergoing HSCT is challenging, damage to endothelial cells is regarded as a common feature of these complications [7-12]. Furthermore, endothelial damage perpetuated by $CD8^+$ CTL has been linked to GVHD and described for the skin and gut [8, 13-15].

Recombinant thrombomodulin (rTM) is composed of the active extracellular domain of TM. Like membrane-bound TM, rTM binds to thrombin to inactivate coagulation [16]. The resulting thrombin-rTM complex activates a protein to produce active protein C (APC) which inactivates factors VIIIa and Va in the presence of protein S, thereby inhibiting further thrombin formation [17, 18]. Therefore, rTM might be useful for transplantation-associated coagulopathy (TAC) after HSCT. Indeed there are some reports on the efficacy of rTM therapies for TAC, including VOD and TMA [19-21]. However, the preventive effects of rTM against aGVHD following HSCT are poorly understood [22].

We measured and compared the levels of cytokines and soluble factors in patients undergoing allogeneic HSCT. The aims of this study were to investigate the role of endothelial cells in the mechanisms underlying the development of aGVHD and to examine the prophylactic use of rTM for aGVHD.

MATERIALS AND METHODS

Subjects

The subjects were 312 patients who underwent allogeneic SCT between June 2011 and March 2015 at 25 institutions in Japan (Table 1). The 179 male and 133 female allogenic SCT patients ranged in age from 7 to 77 years (median: 45 years). Conditioning applied was total body irradiation for 192 and non-total body irradiation for 120. The donor sources were 161 bone marrow transplantations, 67 peripheral blood stem cell transplantations and 84 cord blood transplantations. Written informed consent was obtained from all patients who were registered by faxing documents to Kansai Medical University prior to SCT. The rTM consisting of daily doses of 380 units/kg (Asahi Kasei Pharma, Tokyo, Japan) was administered as a preventive therapy for TAC. This protocol was completed during the days 4 to 14 after HSCT. An anticoagulation regimen of 5000 U heparin 24 h per day was used prior to rTM administration. Heparin was administered to the control groups, replacing rTM. In addition, patients who did not receive the anticoagulant were also included in the control groups.

Measurement of interleukin (IL)-6, tumor necrotizing factor (TNF)α, monocyte-chemotactant protein (MCP)-1, Regulated on activation normally T-cell expressed and secreted (RANTES), high mobility group box 1 (HMGB1), sVascular cell adhesion molecule (VCAM)-1, sE-selectin, sFas ligand (L) and plasminogen activator inhibitor (PAI-1)

Blood samples from patients were collected into tubes containing sodium citrate or tubes without any anticoagulant and the blood samples were allowed to clot at room temperature for a minimum of 1 hour. The serum or citrated plasma was isolated by centrifugation for 20 minutes at 1,000 × g at 4 °C. The serum was divided into aliquots and frozen at -30 °C until use. As a positive control, recombinant products as well as standard solutions provided with the commercial kits were used in each assay. Human IL-6, TNFα, MCP-1, RANTES, sFasL, sVCAM-1, sE-selectin and PAI-1 ELISA kits were purchased from BioSource International, Inc. (Camarillo, California, USA). HMGB1 was measured using HMGB1 ELISA kit II (Shino-test Corp., Kanagawa, Japan). Serum levels of cytokine and soluble factors were measured according to the manufacturer's instructions.

Assessment of platelet-derived microparticle (PDMP)

For PDMP assay, blood samples were collected with a 21-gauge needle from a peripheral vein in vacutainers containing EDTA-ACD (NIPRO Co. Ltd., Japan) to minimize platelet activation. The samples were handled as described in the manufacturer's protocol. The samples were gently

Table 1. Patients and treatment characteristics.

	Allogeneic HSCT
Sex	
Male/Female	179/133
Median age (range)	45 (7-77)
Patient diagnosis at transplantation	
Acute myeloblastic leukemia (AML)	111
Acute lymphoblastic leukemia (ALL)	68
Myelodysplastic syndrome (MDS)	52
Other	81
Conditioning regimen	
TBI-conditioning	192
CY/TBI	63
Flu/Bu/TBI	27
Flu/Mel/TBI	24
VP16/CY/TBI	22
Other	56
Non-TBI-conditioning	120
Flu/Bu	41
Bu/CY	19
Flu/Bu/ATG	16
Other	44
Donor source	
Bone marrow transplantation (BMT)	161
Peripheral blood stem cell transplantation (PBSCT)	67
Cords blood transplantation (CBT)	84
Prophylaxis for GVHD	
FK/sMTX	166
CyA/sMTX	69
FK/mPSL	18
FK/MMF	17
Other	42
Anticoagulant	
No anticoagulant	117
Heparin	64
rTM	131

TBI: total body irradiation; CY: cyclophosphamide; Flu: fludarabine; Bu: busulfan; Mel: melphalan; ATG: anti-thymocyte globulin; GVHD: graft-versus-host disease; FK: tacrolimus; sMTX: short-term methotrexate; CyA: cyclosporine; mPSL: methylprednisolone; MMF: mycophenolate mofetil; rTM: recombinant soluble thrombomodulin.

mixed by inverting the tube once or twice, stored at room temperature for 2-3 hrs and centrifuged at 8,000 g for 5 mins at room temperature. Storage of the samples at room temperature for 2-3 hrs did not affect the PDMP level. Immediately after centrifugation, 200 μl of upper-layer supernatant was collected from 2 ml samples to avoid contamination of the platelets [23] and the samples

were stored at -40 °C until analysis. The PDMP level was measured in duplicate and the mean values were recorded. For the measurement of PDMPs we used an ELISA kit (JIMRO Co. Ltd., Japan) and monoclonal antibodies against glycoprotein CD42b and CD42a (glycoprotein Ib and IX) [23-25].

Statistical analysis

Data were expressed as means ± SD. The statistical significance of differences between the groups was analyzed by chi-square, Newman–Keuls, or Scheffe's tests. The patients without rTM were divided into two subgroups based on their sVCAM-1 levels at day 28 after HSCT. Group A was defined as patients showing a > 1.5-fold increase in their sVCAM-1 levels after HSCT relative to the levels at day 7 after HSCT, while group B was defined as those with a < 1.5-fold increase in their sVCAM-1 levels in the same time frame. The differences between the MCP-1, RANTES, TNFα and sFasL levels in groups A and B were evaluated by analysis of variance (ANOVA). Overall survival (OS) was defined as the time from initial diagnosis to the time of death from any cause or the date when the patient was last known to be alive. Univariate analyses of OS were performed using the Kaplan–Meier product-limit method with a log-rank test and the Cox proportional hazards model, respectively. All statistical analyses were performed using StatFlex (ver. 6) software (Artech, Osaka, Japan). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Table 2 shows changes in various factors with HSCT. Although IL-6 and TNF were increased following HSCT, these fluctuations were not significant. MCP-1 was significantly increased after HSCT and reached a peak on day 14 both with and without rTM. RANTES did not exhibit any significant changes. HMGB1 was decreased after HSCT, and this tendency was significant in patients with rTM. sVCAM-1, sE-selectin, sFasL, PAI-1 and PDMP exhibited significant increases after HSCT, and these tendencies were significant in patients without rTM.

When we examined patients with confirmed complications, the frequencies of aGVHD and VOD were significantly lower in the rTM(+) group, while the frequencies of TMA and HPS were not (Table 3). In addition, the frequencies of elevation of endothelial cell-activation markers such as sVCAM-1, sE-selectin and PAI-1 were significantly higher in the rTM(−) group (Table 3).

We divided the patients without rTM (n = 181) into two subgroups according to the elevation of sVCAM-1 after HSCT. The high sVCAM-1 group showed significant increase in the plasma concentrations of MCP-1, RANTES, TNFα and sFasL relative to baseline (day 0) ($p < 0.05$ or $p < 0.01$; Figure 1A-D), and all four concentrations were significantly higher in the high sVCAM-1 group than in the low sVCAM-1 group at day 28 after HSCT (two-factor ANOVA: $p < 0.01$ or $p < 0.05$). However, the low sVCAM-1 group showed no significant increase in the plasma concentrations of MCP-1, RANTES, TNFα and sFasL (Figure 1A-D).

Figure 2 shows the Kaplan–Meier curves for OS of the patients with or without rTM. The log-rank p-value at 400 days between patients with or without rTM was 0.043, while that at 1000 days was 0.397.

DISCUSSION

Although allogeneic HSCT has made remarkable progress in therapeutic performance, aGVHD remains the most important complication of HSCT [26]. GVHD was originally regarded as an epithelial cell disease, and the three organs mainly involved in aGVHD are the skin, gastrointestinal tract and liver [27]. Donor T-cells can become activated through recognition of host alloantigens and differentiate into effector T-cells that recruit other cell types and lead to local inflammation and target tissue destruction [28]. In the final stage of GVHD, the Fas/FasL system plays a very important role in pathogenesis [3, 6] because the interaction between FasL, expressed on the CTL cell surface and the Fas receptor expressed on the target cell membrane results in initiation of the Fas cell death pathway [29]. However, it has been suggested that vascular endothelial vulnerability and endothelial dysfunction are also involved in the pathogenesis of GVHD [9-12]. Specifically, endothelial cells become a target for CTL resulting in induction of endothelial cell apoptosis

Table 2. Changes in various factors with HSCT.

Cytokine/Factor	day 0	day 7	day 14	day 28
All patients (n=312)				
IL-6 (pg/ml)	14.3 ± 22.8	47.6 ± 123.9	61.3 ± 155.2	11.5 ± 12.4
TNFα (ng/ml)	15.6 ± 3.5	16.1 ± 4.2	17.3 ± 4.7	18.3 ± 5.2
MCP-1 (pg/ml)	511 ± 191	822 ± 389*	937 ± 752**	474 ± 156
RANTES (ng/ml)	44.7 ± 18.5	41.2 ± 12.5	45.6 ± 15.1	49.8 ± 15.6
HMGB1 (ng/ml)	11.3 ± 3.3	8.6 ± 2.4	7.5 ± 2.1*	6.7 ± 2.7*
sVCAM-1 (pg/ml)	767 ± 384	876 ± 393	895 ± 441	1,022 ± 368*
sE-selectin (pg/ml)	74.9 ± 37.1	72.8 ± 32.9	74.3 ± 31.6	79.6 ± 35.5
sFasL (ng/ml)	0.12 ± 0.06	0.14 ± 0.06	0.18 ± 0.09	0.21 ± 0.08*
PAI-1 (ng/ml)	15.3 ± 7.0	15.5 ± 6.2	18.4 ± 8.8*	19.5 ± 8.3*
PDMP (U/ml)	8.5 ± 3.6	8.8 ± 3.3	10.1 ± 3.9	13.7 ± 4.7**
Patients without rTM (n = 181)				
IL-6 (pg/ml)	15.2 ± 22.6	73.5 ± 181.5	49.8 ± 104.3	12.7 ± 13.4
TNFα (ng/ml)	15.9 ± 3.8	17.9 ± 4.5	19.8 ± 5.2	25.6 ± 6.7*
MCP-1 (pg/ml)	502 ± 186	861 ± 384*	977 ± 741**	568 ± 159
RANTES (ng/ml)	45.6 ± 19.2	44.7 ± 12.8	50.6 ± 16.6	56.3 ± 17.2*
HMGB1 (ng/ml)	11.3 ± 3.4	9.4 ± 3.1	8.7 ± 2.6*	7.2 ± 2.8*
sVCAM-1 (pg/ml)	783 ± 392	945 ± 442	1,084 ± 521*	1,213 ± 511**
sE-selectin (pg/ml)	76.1 ± 38.2	80.4 ± 39.6	86.7 ± 38.9*	92.4 ± 43.2**
sFasL (ng/ml)	0.12 ± 0.08	0.16 ± 0.07	0.21 ± 0.11*	0.27 ± 0.13**
PAI-1 (ng/ml)	16.1 ± 7.2	19.3 ± 7.8	24.5 ± 9.6*	28.3 ± 10.1**
PDMP (U/ml)	8.7 ± 3.2	9.9 ± 3.6	13.2 ± 4.5*	20.4 ± 4.9**
Patients with rTM (n = 131)				
IL-6 (pg/ml)	13.7 ± 26.9	29.9 ± 57.1	85.7 ± 203.3	10.8 ± 11.9
TNFα (ng/ml)	15.4 ± 3.3	14.6 ± 3.9	14.7 ± 3.8	13.0 ± 4.5
MCP-1 (pg/ml)	518 ± 197	773 ± 396*	905 ± 760*	395 ± 154
RANTES (ng/ml)	43.8 ± 17.1	36.7 ± 12.1	40.4 ± 14.3	44.0 ± 12.0
HMGB1 (ng/ml)	11.2 ± 3.2	7.2 ± 1.7*	6.8 ± 1.9*	5.0 ± 2.6**
sVCAM-1 (pg/ml)	751 ± 375	811 ± 340	724 ± 335	640 ± 211
sE-selectin (pg/ml)	71.4 ± 35.5	65.7 ± 28.2	61.0 ± 22.5	62.1 ± 26.0
sFasL (ng/ml)	0.11 ± 0.05	0.13 ± 0.04	0.15 ± 0.07	0.16 ± 0.04
PAI-1 (ng/ml)	14.5 ± 6.8	12.7 ± 4.8	11.9 ± 7.2	10.8 ± 6.1*
PDMP (U/ml)	8.4 ± 3.9	7.6 ± 3.0	7.1 ± 3.2	7.3 ± 4.5

Data represent the means ± S.D. IL-6: interleukin-6; TNFα: tumor necrotizing factor α; MCP-1: monocyte-chemotactant protein-1; RANTES: regulated on activation normally T-cell expressed and secreted; HMGB1: high mobility group box 1; sVCAM-1: soluble vascular cell adhesion molecule-1; sE-selectin: soluble E-selectin; sFasL: soluble Fas ligand; PAI-1: plasminogen activator inhibitor; PDMP: platelet-derived microparticle; rTM: recombinant soluble thrombomodulin. The p values are for before (day 0) vs. after (day 7, 14, and 28) HSCT. *: $p < 0.05$, **: $p < 0.01$.

by FasL on the CTL surface [30, 31]. Thus, damage to endothelial cells is regarded as a common feature of the vascular complications in patients undergoing allogeneic HSCT [8]. In the present study, we found significant increase in the levels of some endothelial dysfunction markers including sVCAM-1, sE-selectin and PAI-1 after HSCT, and these tendencies were significant in patients

Table 3. The frequencies of complications and EC-activation markers in patients with or without rTM.

	rTM (–) (n = 181)	rTM (+) (n = 131)	p value
Complication			
aGVHD	98	42	0.0153
VOD	37	14	0.0494
TMA	27	10	0.0795
HPS	16	6	0.1750
*Elevation of EC-activation marker**			
sVCAM-1	85	21	< 0.0001
sE-selectin	73	24	0.0022
PAI-1	76	23	0.0008

rTM: recombinant soluble thrombomodulin; aGVHD: acute graft-versus-host disease; VOD: veno-occlusive disease; TMA: thrombotic microangiopathy; HPS: hemophagocytic syndrome; sVCAM-1: soluble vascular cell adhesion molecule; PAI-1: plasminogen activator inhibitor.

*: This was determined by proportion of a mean value in day 14 compared to the value in day 0 in patients without rTM. The significant elevation is sVCAM-1 > 1.38, sE-selectin > 1.14 and PAI-1 > 1.52, respectively.

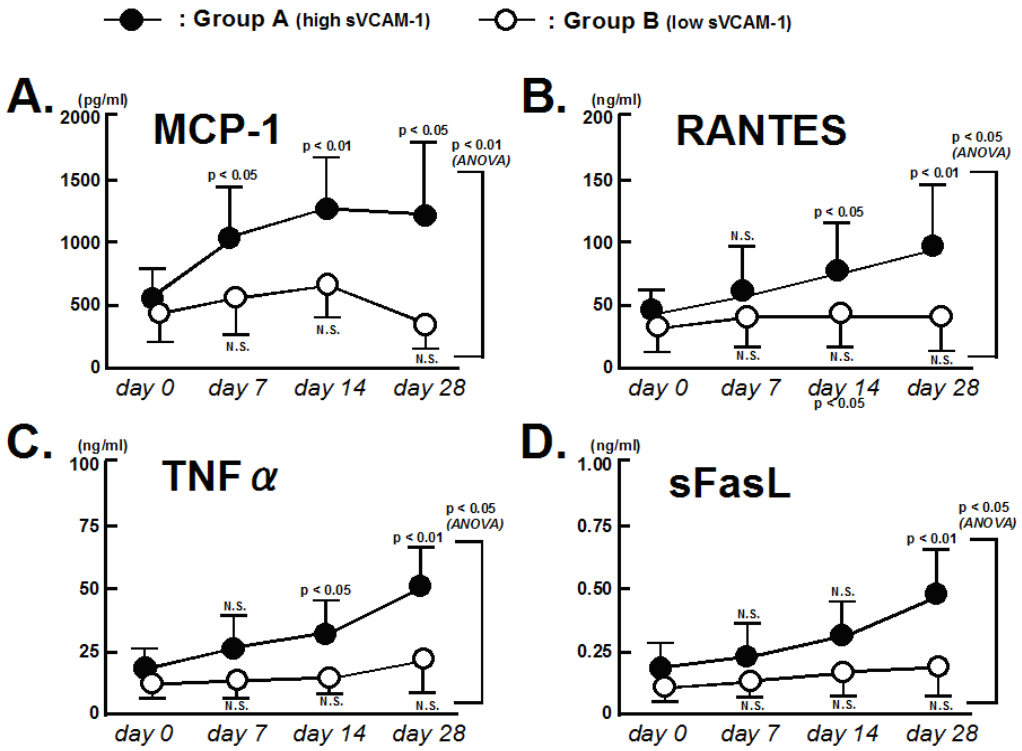

Figure 1. Changes in MCP-1, RANTES, TNFα and sFasL in patients without rTM, with and without significant elevation of sVCAM-1 after HSCT. Values are presented as mean ± SD. P-values are for comparison with each baseline parameter (day 0 vs 7, 14 and 28). ANOVA: Group A versus Group B MCP-1: monocyte-chemotactant protein-1; RANTES: regulated on activation normally T-cell expressed and secreted; TNFα: tumor necrotizing factor α; sFasL: soluble Fas ligand.

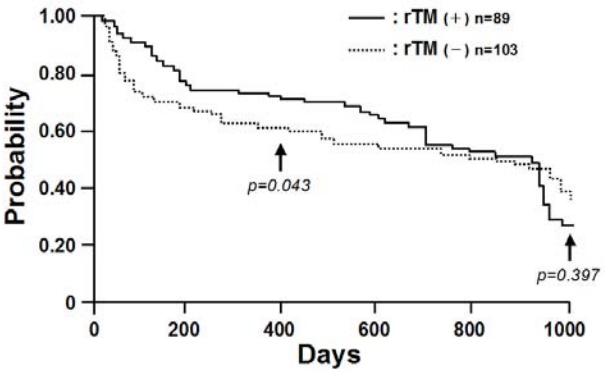

Figure 2. Kaplan-Meier curves for overall survival of the patients with or without rTM. rTM: recombinant soluble thrombomodulin.

without rTM. These findings suggest that rTM can lessen the endothelial dysfunction after HSCT.

There are many causes for HSCT-associated complications [26]. Damage induced by the conditioning regimen and infections leads to the release of diverse cytokines that are responsible for an inflammatory process, thereby enhancing the GVHD reaction [9]. In particular, we regard HMGB1 as being important [32, 33]. HMGB1 is released upon tissue damage as an endogenous damage-associated molecular pattern and is actively produced by immune cells [26, 34]. Previous reports have suggested relationships between HMGB1 and HSCT-related complications or TMA-associated aGVHD [35, 36]. In particular, Inoue *et al.* [36] reported that rTM which possesses the ability to neutralize HMGB1 was very useful for aGVHD. In the present study HMGB1 was decreased after HSCT, and this tendency was significant in patients with rTM.

There are some previous reports on the efficacy of rTM for HSCT-related complications such as VOD and TMA [19-21]. However, the useful effects of rTM for aGVHD following HSCT are poorly understood [22, 32]. Recently Ikezoe *et al.* [22] demonstrated that rTM alleviated aGVHD in association with decreased plasma levels of inflammatory cytokines and HMGB1 in a murine aGVHD model. Furthermore, they concluded that an increase in the proportion of regulatory T cells in the spleen was associated with these rTM-related effects for aGVHD [22]. In the present study, the frequencies of aGVHD and VOD were significantly lower in the rTM(+) group, while those of TMA and HPS were not. In addition, the frequencies of elevation of endothelial cell-activation markers were significantly higher in the rTM(−) group. These results suggest that rTM can lessen the endothelial dysfunction and prevent aGVHD after HSCT.

Chemokines play an important role in leukocyte trafficking and are also involved in a variety of inflammatory and infectious conditions including GVHD [37-39]. In particular, MCP-1 and RANTES are closely related to the pathophysiology of aGVHD after HSCT [15, 29, 30, 40-45]. Therefore, we analyzed the relationships between endothelial dysfunction and MCP-1/RANTES after HSCT. The high sVCAM-1 group showed significant increases in the plasma concentrations of MCP-1, RANTES, TNFα, and sFasL relative to baseline (day 0), and all four concentrations were significantly higher in the high sVCAM-1 group than in the low sVCAM-1 group at day 28 after HSCT. However, the low sVCAM-1 group did not show significant increase in those concentrations. These results suggest that endothelial dysfunction is related to aGVHD, and that rTM can lessen this dysfunction after HSCT. Indeed, Kaplan–Meier curves for OS of the patients with and without rTM exhibited a significant preventive effect of rTM for aGVHD at 400 days, but not at 1000 days, after HSCT.

CONCLUSION

Our findings have two potential implications. First, we have shown that endothelial dysfunction is related to aGVHD after allogeneic HSCT. Second, we have described the prophylactic effect of rTM for aGVHD after allogeneic HSCT. Nevertheless, our study has some limitations: this was not a randomized study and we were unable to determine the relationship between the effects of rTM and the conditioning regimen, which can be an important determinant for the development of transplant-related complications. Further confirmation of these observations in prospective and randomized studies is necessary.

CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest to report for this work.

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