Transplant Immunology 53 (2019) 28–33

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Effects of recombinant thrombomodulin therapy and soluble human leukocyte antigen-G levels during hematopoietic stem cell transplantation

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ARTICLE INFO

Keywords:
sHLA-G
Stem cell transplantation
rTM
aGVHD

ABSTRACT

Background: Conditioning chemotherapies for hematopoietic stem cell transplantation (HSCT), especially those that include total body irradiation, can result in serious complications such as graft-versus-host disease (GVHD). Human leukocyte antigen G (HLA-G) is a non-classical class I molecule with multiple immunoregulatory functions.

Methods: We measured interleukin (IL)-10, transforming growth factor (TGF)β_1, and soluble HLA-G (sHLA-G) in HSCT patients and examined the relationship between sHLA-G levels and acute GVHD (aGVHD). Additionally, we investigated the effect of recombinant soluble thrombomodulin (rTM) therapy on sHLA-G levels. Our study cohort included 135 patients who underwent allogeneic HSCT at several institutions in Japan.

Results: Serum levels of IL-10 and TGFβ_1 exhibited no significant changes following HSCT. In contrast, levels of sHLA-G were significantly increased at days 21 and 28 post-HSCT. For patients with confirmed complications, the frequency of aGVHD was significantly lower in those with a $>$ 2.8-fold increase in sHLA-G levels at day 28 relative to day 7 post-HSCT. sHLA-G levels in patients who received rTM therapy were significantly higher at days 21 and 28 post-HSCT compared with those in patients who did not receive rTM therapy.

Conclusion: These data suggest that HLA-G/sHLA-G participate in prevention of GVHD, and that rTM may prevent aGVHD following HSCT by promoting elevation of sHLA-G.

1. Introduction

Although hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for patients with hematologic malignancies, serious transplant-related complications, including graft-versus-host disease (GVHD), occur frequently [1,2]. Several studies have indicated involvement of the Fas/FasL system or abnormalities of certain cytokines such as interleukin (IL)-10 and transforming growth factor β_1 (TGFβ_1) in the pathogenesis of GVHD [2–5]. Moreover, endothelial damage caused by $CD8^+$ cytotoxic T lymphocytes (CTLs) has been linked to GVHD and found in both the skin and gut [6–8]. An important factor associated with the success of HSCT is the human leukocyte antigen (HLA) type. Thus, improving donor selection strategies is an important goal. Furthermore, the HLA type is highly associated with the development of GVHD [2].

HLA system loci are located on the short arm of chromosome six and subdivided into HLA class I and class II genes. HLA class I genes can be further subdivided into class Ia (HLA-A, -B, and -C) and class Ib (HLA-E, -F, and -G) genes [9,10]. The HLA class Ia loci are highly polymorphic and expressed on the surface of many somatic cells [9,10]. In contrast, HLA class Ib loci are much less polymorphic [11]. HLA-G rose to prominence as an unknown HLA molecule identified on placental trophoblasts, that do not express HLA class I or II molecules [12]. In addition, some reports have suggested that HLA-G is expressed on the surface of many non-placental cells. Membrane-anchored HLA-G and its soluble form (sHLA-G) have been demonstrated to perform multiple functions that promote immune tolerance to allogeneic molecules [13–15]. In particular, the association between HLA-G and GVHD has become a central focus in HSCT [16–19].

Recombinant thrombomodulin (rTM), which is the active extracellular domain of thrombomodulin, binds to thrombin to inactivate coagulation (Fig. 1) [20]. The resulting thrombin-rTM complex

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https://doi.org/10.1016/j.trim.2018.12.001
Received 7 November 2018; Received in revised form 9 December 2018; Accepted 9 December 2018
Available online 10 December 2018
0966-3274/

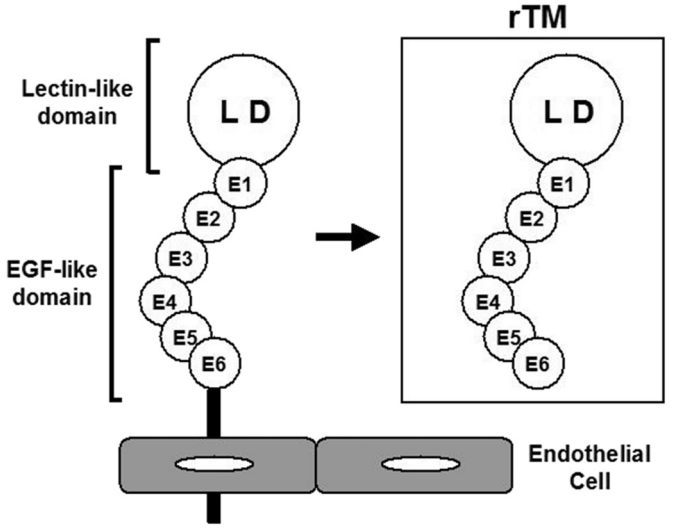

Fig. 1. Schema of TM and rTM structures. LD: lectin-like domain; E1-E6: EGF-like domain 1–6; rTM: recombinant thrombomodulin. rTM has the same structure as the extracellular domain (lectin-like and EGF-like domains) of endothelial TM.

produces activated protein C (APC) that inactivates factors VIIIa and Va in the presence of protein S, thereby inhibiting further thrombin formation [21–23]. In addition, rTM binds to and inhibit the high mobility group box 1 (HMGB1) protein, which is one of the causes of disseminated intravascular coagulation [22]. HMGB1 is also mobilized from apoptotic and necrotic cells by conditioning regimens for HSCT [24,25]. Therefore, rTM might be useful for treating transplantation-associated coagulopathy (TAC) following HSCT. Indeed, there have been some reports on the efficacy of rTM therapies for TACs, including veno-occlusive disease (VOD) and thrombotic microangiopathy (TMA) [26–33]. In addition, the efficacy of rTM in treating acute GVHD (aGVHD) following HSCT has been demonstrated recently [34,35].

Here, we measured and compared the levels of IL-10, TGF-β_1, and sHLA-G in patients who underwent allogeneic HSCT with or without rTM treatment. The aims of this study were to investigate the role of rTM and sHLA-G in the pathophysiology underlying the development of GVHD and to assess the potential for prophylactic use of rTM for GVHD treatment.

2. Materials and methods

2.1. Subjects

The subjects in this study were patients who underwent allogeneic HSCT between July 2012 and September 2015 at several institutions in Japan (Table 1). Written informed consent was obtained from all patients, who were registered by faxing documents to Kansai Medical University prior to HSCT. Although some data from this cohort have been published previously [25,35], the present study included unpublished or partially published biomarker data as well as an additional 135 patients who only recently provided informed consent. These allogeneic HSCT patients (82 males and 53 females) ranged in age from 21 to 73 years (median: 45 years). The applied conditioning regimen was total body irradiation for 90 patients and non-total body irradiation for 45 patients. The donor sources included 76 bone marrow transplants, 28 peripheral blood stem cell transplants, and 31 cord blood transplants. rTM (Asahi Kasei Pharma, Tokyo, Japan) was administered at a daily dose of 380 U/kg, as a preventive therapy for TAC. This protocol was completed from days 4 to 14 following HSCT. A preparative anticoagulation regimen of 5000 U heparin (24 h per day) was used prior to rTM administration. Control groups were administered additional heparin instead of rTM. In addition, patients who received no anticoagulant were included in control groups.

Table 1
Patient and treatment characteristics. 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.

	Allogeneic HSCT
Sex: Male/Female	82 /53
Median age (range)	45 (21–73)
Patient diagnosis at transplantation:	
Acute myeloblastic leukemia (AML)	69
Acute lymphoblastic leukemia (ALL)	40
Myelodysplastic syndrome (MDS)	26
Other	
Conditioning regimen:	
TBI-conditioning:	90
CY/TBI	41
Flu/Bu/TBI	10
Flu/Mel/TBI	9
VP16/CY/TBI	7
Other	23
Non-TBI-conditioning:	45
Flu/Bu	21
Bu/CY	8
Flu/Bu/ATG	7
Other	9
Donor source:	
Bone marrow transplantation (BMT)	76
Peripheral blood stem cell transplantation (PBSCT)	28
Cords blood transplantation (CBT)	31
Prophylaxis for GVHD:	
FK/sMTX	74
CyA/sMTX	29
FK/mPSL	17
FK/MMF	6
Other	9
Anticoagulant:	
No anticoagulant	37
Heparin (day 0-day 14)	26
rTM (day 0-day 3: heparin, day 4-day 14: rTM)	72

2.2. Complications following HSCT

When complications were reported following HSCT, the presence of aGVHD, VOD, and TMA were determined by the chief physician in each facility with reference to previous reports concerning aGVHD [36,37], VOD [38,39], and TMA [40].

2.3. Biomarker quantitation

Patient blood samples were collected into tubes containing sodium citrate or empty tubes. The latter samples were allowed to clot at room temperature for a minimum of 1 h. The serum or citrated plasma was centrifuged for 20 min at 1000 ×*g* at 4 °C. Serum samples were aliquoted and stored at −30 °C until use. As positive controls, recombinant proteins and standard solutions provided with commercial kits were used in each assay. Plasma concentrations of IL-10 and TGF-β_1 were measured using monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Inc., Camarillo, CA, USA). An ELISA kit from BioVender, Inc. (Brno, Czech Republic) was used to quantitate plasma sHLA-G. In the BioVendor sHLA-G ELISA, calibrators and samples were incubated in microplate wells pre-coated with a monoclonal anti-sHLA-G antibody. After incubation and washing, a monoclonal anti-human β_2-microglobulin antibody labelled with horseradish peroxidase was added to the wells, followed by incubation with captured sHLA-G [41,42]. After reacting with the substrate solution, the absorbance of the resulting colorized product was measured.

The calibration range of sHLA-G was 3.91–125 U/ml. The manufacturer's instructions indicated that accurate measurement of TGF-β_1 was dependent on avoiding platelet activation. Therefore, precautions to avoid platelet activation were undertaken. All kits were used in accordance with the manufacturer instructions.

2.4. Statistical analysis

Data are expressed as means ± standard deviations. The statistical significance of differences between groups was analyzed by chi-squared Newman-Keuls, or Scheffe's tests. Patients were stratified into two groups based on their sHLA-G levels following HSCT. Receiver operating characteristic (ROC) curve analysis was used to estimate the optimal cutoff value for changes in sHLA-G levels. Group A was defined as patients with a > 2.8-fold increase in the sHLA-G concentration at day 28 post-HSCT relative to day 7, whereas group B was defined as those with a < 2.8-fold increase in sHLA-G over the same time period. Differences in IL-10, TGF-β_1, and sHLA-G levels of rTM(+) and rTM(−) patients were evaluated by analysis of variance (ANOVA). In addition, logistic regression analysis was performed to assess whether multivaliate factors were associated with the presence of aGVHD. All statistical analyses were performed using StatFlex v. 6 software (Artech, Osaka, Japan). Values of $p < .05$ were considered as statistically significant.

3. Results

Fig. 2 shows changes in serum levels of IL-10, TGF-β_1, and sHLA-G following HSCT. Although serum IL-10 and TGF-β_1 concentrations showed no significant changes following HSCT, sHLA-G levels were significantly increased at days 21 and 28.

We stratified patients into two groups based on changes in sHLA-G levels following HSCT. ROC curve analysis was used to estimate the optimal cutoff value for the sHLA-G level. Group A was defined as patients with a > 2.8-fold increase in sHLA-G at day 28 post-HSCT relative to day 7, whereas group B was defined as those with a < 2.8-fold increase over the same time period. When we examined patients with confirmed complications, the frequency of aGVHD (grade I and II-IV), but notVOD or TMA, was significantly lower in group A (Table 2). In particular, aGVHD at days 22–60 was more significant.

Table 2
Frequency of complications in HSCT patients with or without high sHLA-G levels. aGVHD: acute graft-versus-host disease; VOD: veno-occlusive disease; TMA: thrombotic microangiopathy.*

	Group A*	Group B*	*p* value
n	65	70	–
Complication			
aGVHD	23	52	0.0369
day 7–21	4	6	0.3677
day 22–60	19	46	0.0281
Grade I	5	13	0.0224
Grade II-IV	18	39	0.0425
VOD	5	8	0.3124
TMA	7	14	0.2719

* Patients were stratified into two groups based on changes in serum sHLA-G levels after HSCT. Receiver operating characteristic (ROC) curve analysis was used to estimate the optimal cutoff value for the sHLA-G level. Group A was defined as patients showing a > 2.8-fold increase in sHLA-G at day 28 post-HSCT relative to day 7, whereas group B was defined as those with a < 2.8-fold increase over the same time period.

Table 3 shows logistic regression analysis of the presence of aGVHD. In the presence of aGVHD, the odds ratio (95%CI) for rTM–positive and sHLA-G high were 0.163 (0.096–0.312) ($p = .0009$) and 0.194

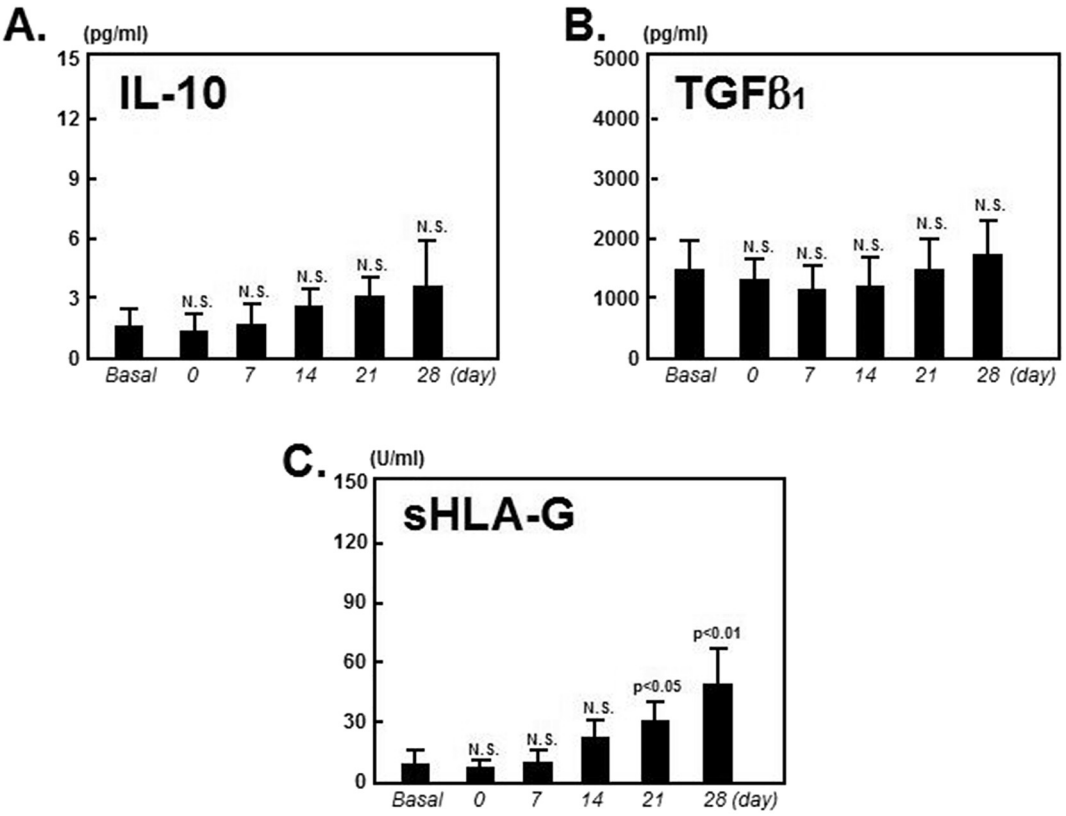

Fig. 2. Changes in serum biomarker abundance following HSCT. Data represent means ± standard deviation. IL-10: interleukin-10; TGFβ1: transforming growth factor β1; sHLA-G: soluble human leukocyte antigen-G. *p*-Values are shown for the comparisons of pre-HSCT (day 0) versus post-HSCT (days 7, 14, 21, and 28). N.S.: not significant.

Table 3
Logistic regression analysis of the presence of aGVHD. rTM: recombinant thrombomodulin; BMT: bone marrow transplantation; PBSCT: peripheral blood stem cell transplantation; CBT: cord blood transplantation; sHLA-G: soluble human leukocyte antigen-G; IL-10: interleukin-10.

Facor	Point Estimate	Odds ratio 95%CI	P value
rTM-positive	0.163	0.096–0.312	0.0009*
Men	0.867	0.471–1.536	0.6173
Age	0.839	0.675–1.014	0.1459
BMT	0.975	0.479–2.038	0.9713
PBSCT	1.125	0.512–3.359	0.5127
CBT	1.445	0.439–4.757	0.6325
sHLA-G high	0.194	0.112–0.375	0.0027*
IL-10 high	0.254	0.191–0.531	0.0988

* p-Value is statistically significant.

(0.112–0.375) (p = .0027), respectively. In contrast, male, age, BMT, PBSCT, CBT, and IL-10 high showed no significant associations.

Finally, we stratified patients into two groups based on whether they received rTM therapy following HSCT. There were no significant differences in serum IL-10 and TGF-β_1 levels between rTM-treated and non-rTM-treated patients. However, sHLA-G levels were significantly different between rTM-treated (n = 72) and non-rTM-treated (n = 63) patients (Fig. 3). sHLA-G levels in patients who received rTM therapy were significantly higher at days 21 and 28 post-HSCT compared with those in patients who did not receive rTM therapy (two-way ANOVA; $p < .05$).

4. Discussion

GVHD is a common complication following HSCT, which depends on the composition of immune cells derived from the donor stem cells [2]. Donor T cells can activate through recognition of host alloantigens and differentiate into effector T cells. These effector T cells recruit other cell types, leading to local inflammation and targeted tissue destruction [43]. The levels of several cytokines and chemokines increase after HSCT, promoting the development of aGVHD [44,45]. In addition to cytokines and chemokines, some reports have suggested that HLA-G and/or sHLA-G are major factors in development of aGVHD [16–19]. Liu et al. [17] reported that sHLA-G5 levels might predict the occurrence and severity of aGVHD, which may be helpful to develop personalized prophylactic therapies against aGVHD and improve survival following HSCT. Furthermore, Kordelas et al. [19] reported that elevated sHLA-G levels were associated with severe GVHD, and that sHLA-G had a tolerance-inducing function. In the present study, serum sHLA-G levels were significantly increased at days 21 and 28 following HSCT. These results suggest that elevated sHLA-G contributes to the pathophysiology of aGVHD following HSCT.

sHLA-G is an immunosuppressive molecule that induces apoptosis of activated CD8-positive T cells and down-regulates CD4-positive T cell proliferation [46]. In addition, this molecule inhibits dendritic and antigen-presenting cell functions, and induces regulatory T cell differentiation, resulting in strong immunosuppressive effects [47,48]. HLA-G does not engage T-cell receptors and predominantly binds binds to three inhibitory receptors: immunoglobulin-like transcript (ILT)2, ILT4, and killer immunoglobulin-like receptor (KIR) 2DL4 [15,47]. HLA-G has seven isoforms, of which the major isoforms are HLA-G1 and HLA-G5 [49]. HLA-G5 is a soluble isoform, and HLA-G5 expression and secretion by mesenchymal stem cells has been demonstrated to contribute to inhibition of NK and allogeneic T cell responses [48,50,51]. Furthermore, it is thought that HLA-G restrains NK cell-mediated tissue injury, because HLA-G is a ligand for the three inhibitory receptors (ILT2, ILT4, and KIR2DL4) expressed on the surface of almost all NK cells [49,52]. These findings suggest that HLA-G/sHLA-G play an important role in the prevention of GVHD. In the present study, the frequency of aGVHD was significantly lower in patients with increasing

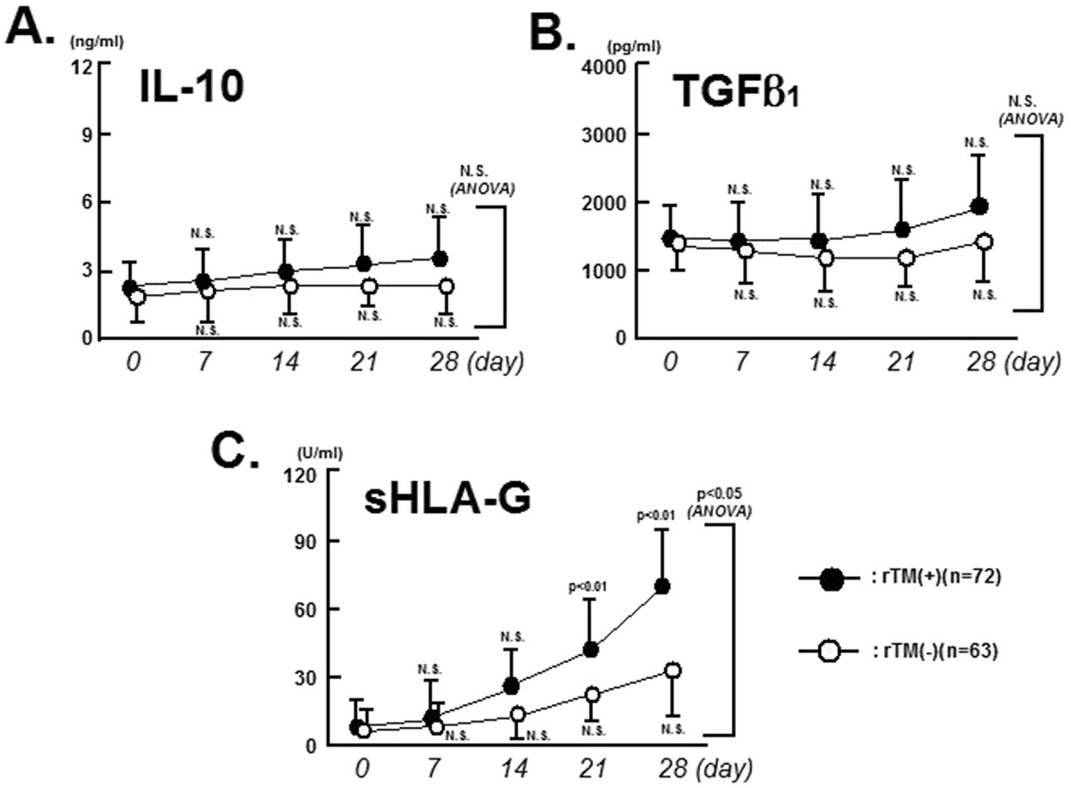

Fig. 3. Changes in serum IL-10, TGFβ1, and sHLA-G levels in patients with or without rTM therapy following HSCT. Values are presented as means ± standard deviation. p-Values are shown for comparisons between each parameter at baseline and post-HSCT (day 0 vs days 7, 14, 21, and 28). ANOVA: rTM-treated versus non-rTM-treated patients. IL-10: interleukin-10; TGFβ1: transforming growth factor β1; sHLA-G: soluble human leukocyte antigen-G. N.S.: not significant.

levels of serum sHLA-G at day 28 relative to day 7 post-HSCT. Although aGVHD at days 7–21 did not show significant differences, aGVHD at days 22–60 showed obvious significant differences. Grade I and II-IV aGVHD also showed significant differences. Thus, our results confirmed the suppressive effect of sHLA-G on aGVHD, which is in agreement with previous reports [16–19].

Another interesting result of our study was the significant elevation of sHLA-G observed in patients treated with rTM. The utility of rTM in treating complications following HSCT has been reported in both patients with VOD/TMA and aGVHD [26–35]. The pathogenesis of GVHD involves vascular endothelial vulnerability and endothelial dysfunction [13–16,53,54]. Specifically, endothelial cells become a target of CTLs, resulting in the induction of endothelial cell apoptosis [55,56]. The effect of rTM on GVHD appears to be medicated by reduction of endothelial damage in patients who undergo HSCT. However, in the present study, we found that the levels of sHLA-G were significantly increased after HSCT in patients who received rTM. Furthermore, in logistic regression analysis of the presence of aGVHD, the odds ratio (95%CI) for rTM–positive and sHLA-G high were 0.163 (0.096–0.312) (p = .0009) and 0.194 (0.112–0.375) (p = .0027), respectively. In contrast, male, age, BMT, PBSCT, CBT, and IL-10 high showed no significant associations. These findings suggest that rTM prevents GVHD through effects on sHLA-G, which are unrelated to endothelial dysfunction.

The mechanism through which rTM causes elevation of sHLA-G is still unclear in many respects. Damage induced by conditioning regimens and infections leads to the release of diverse cytokines that are responsible for the inflammatory process, thereby enhancing GVHD [57]. We previously regarded HMGB1 as being important in this process [25,35]. Upon tissue damage, HMGB1 is released as an endogenous damage-associated molecular pattern and actively produced by immune cells [58,59]. Previous reports have suggested relationships between HMGB1 and HSCT-related complications such as aGVHD [35,60]. Importantly, grafted stem cells interact with mesenchymal stromal cells (MSCs) that play a fundamental role in immune modulation. In addition, sHLA-G is secreted from MSCs after interaction between stem cells and MSCs [61]. Lofti et al. [62] demonstrated that HMGB1 not only attracted MSCs, but also enhanced MSC proliferation and inhibited the ability of MSCs to produce immunosuppressive molecules. Their report suggested that overabundance of HMGB1 inhibited production of sHLA-G by MSCs. Thus, rTM appears to cause increased levels of sHLA-G through downregulation of HMGB1. However, we could not test hypothesia in the present study. Nonetheless, the frequency of aGVHD was significantly lower in patients with high sHLA-G, and rTM treatment caused significant elevation of sHLA-G levels. These results suggest that rTM induces elevation of sHLA-G and prevents aGVHD following HSCT by such elevation, although this hypothesis requires experimental confirmation.

In conclusion, our findings have two potential implications. First, we have shown that serum sHLA-G is elevated following allogeneic HSCT. Second, we have described the effect of rTM on serum sHLA-G following allogeneic HSCT. Our study had some limitations. The study was not randomized and we were unable to determine the relationships between the effects of rTM and other major factors, which might be important determinants for changes in sHLA-G levels. Further confirmation of our observations in fundamental and clinical studies is necessary.

Conflict of interest statement

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

Acknowledgements

This work was partly supported by a grant from the Japan Foundation of Cardiovascular Research and by a Research Grant for Advanced Medical Care from the Ministry of Health and Welfare of Japan. We thank Edanz Group for editing a draft of this manuscript.

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