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Evaluation of Angiopoietins and Cell-Derived Microparticles after Stem Cell Transplantation

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ABSTRACT

Although stem cell transplantation (SCT) is being used for hematopoietic reconstitution following high-dose chemotherapy for malignancy, it involves certain serious transplant-related complications such as graft-versus-host disease (GVHD). Angiopoietins play important roles in angiogenesis. However, the role of angiopoietins after SCT is poorly understood. In this study, 52 patients underwent SCT; 26 patients received allogeneic SCT, while the remaining 26 received autologous SCT. In 48 of 52 patients, levels of angiopoietins, cytokines, and soluble factors were measured by enzyme-linked immunosorbent assay. Soluble Fas ligand (sFasL) and endothelial cell-derived microparticle (EDMP) exhibited significant elevation in the early phase (2-3 weeks) after SCT. In addition, the elevation of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and sIL-2 receptor (sIL-2R), which are GVHD markers after allogeneic SCT was observed. The level of angiopoietin (Ang)-2 in allogeneic SCT continued to increase for up to 4 weeks, although the level of Ang-1 did not show significant changes. The patients with high Ang-2 exhibited significant increase of sFasL and EDMP compared with those with low Ang-2. In addition, the patients with high-grade GVHD exhibited a significant increase in Ang-2 compared to patients with low-grade GVHD. In the in vitro experiment using endothelial cells, the suppressive effect of Ang-1 on EDMP generation by TNF-α was partially inhibited by the addition of Ang-2. Furthermore, multivariate regression analysis showed that EDMP and sFasL were significant factors in Ang-2 elevation. Our results suggest that Ang-2 generation after allogeneic SCT relates to GVHD.

KEY WORDS

Angiopoietin • GVHD • stem cell transplantation • sFas • endothelial cell-derived microparticle

INTRODUCTION

Although stem cell transplantation (SCT) is being used for hematopoietic reconstitution following high-dose chemotherapy for malignancy, it involves some serious transplant-related complications [1,2]. For example, graft-versus-host disease (GVHD), and vascular disorders, such as veno-occlusive disease, pulmonary vasculopathy, thrombotic microangiopathy (TMA), and capillary leak syndrome [3-5]. Although the complex pathophysiology of acute GVHD (aGVHD) involves the conditioning regimen, cytokines, nitric oxide, and non-T effector cells, the cytolytic activity of donor T cells is essential for the development of GVHD activity [6,7]. The cytolytic activity of cytotoxic T-lymphocyte (CTL) is primarily mediated through certain effector mechanisms such as the Fas/Fas ligand (FasL) and perforin/granzyme pathways [8,9]. Interaction of FasL, expressed on the CTL cell surface, with the Fas receptor on the target cell membrane results in the initiation of the Fas cell death pathway [10]. Recent accumulating evidence indicates that the Fas/FasL system is implicated in the pathogenesis of aGVHD [7,11-13].

Cellular microparticles are fragments that shed almost spontaneously from the plasma membrane blebs of virtually all cell types when subjected to

a number of stress conditions [14,15]. In addition, these microparticles have more recently been shown to reflect in vitro cell stimulation, and testify to cellular activation and/or tissue degeneration occurring in vivo under various pathophysiologic conditions [14,15]. Thus, there is a possibility that the cellular microparticles exhibit a dynamic change after SCT [16]. In contrast, diagnosing vascular complications in patients undergoing SCT is challenging, and damage to endothelial cells is regarded as the common feature of these complications [17,18]. Furthermore, endothelial damage, perpetuated by $CD8^+$ CTL, has been linked to GVHD, which is described in the skin and gut [18-22].

Angiopoietin-1 (Ang-1) is a ligand of the endothelial-specific tyrosine-kinese receptor Tie2, and is an essential mediator of angiogenesis [23-25]. Ang-1 is also an endothelial survival factor [26], and was recently shown to protect blood vessels against plasma leakage in vivo and to inhibit endothelial permeability in vitro [27-29]. As well, Ang-1 inhibits leukocyte adhesion to vascular endothelium and reduces the expression of tissue factor and various adhesion molecules in endothelial cells stimulated by inflammatory cytokines [29-32]. Ang-2, on the other hand, has been proposed as a natural antagonist of Ang-1, promoting an apoptosis of endothelial cell. Thus, angiopoietins play an important role in a neovasculization and endothelial abnormalities [33-35]. However, the role of angiopoietins after SCT is poorly understood.

We measured and compared levels of angiopoietins, cytokines, and soluble factors in patients undergoing SCT. The results suggested that angiopoietins play a unique role after SCT.

MATERIALS AND METHODS

Patients

The subjects were 52 patients who underwent SCT between August 2001 and June 2007 at the institution of residence. In all, 26 patients received allogeneic SCT, whereas the remaining 26 received autologous SCT (Table 1). The 12 male and 14 female allogeneic SCT patients ranged in age from 6 to 70 years (median: 32 years), and the 14 male and 12 female autologous SCT patients ranged in age from 36 to 71 years (median: 53 years). Patient diagnoses consisted of 7 acute myeloid leukemia, 3 acute promyelocytic leukemia, 6 acute lymphoblastic leukemia, 2 chronic myeloid leukemia, 5 myelodysplastic syndrome, 13 diffuse large B cell lymphoma, 5 follicular cell lymphoma, 7 multiple myeloma, and 4 others. Conditioning applied was: total body irradiation (TBI) for 15 and non-TBI for 37. For allogeneic SCT, prophylaxis included cyclosporine for 26 patients with GVHD. The donor sources were 6 bone marrow transplantations, 12 peripheral blood SCTs, and 8 cord blood transplantations (Table 2). Written informed consent was obtained from all the patients.

Table 1. *Patients and Treatment Characteristics*

	Allogeneic SCT	Autologous SCT
Sex		
Male/female	**12/14**	**14/12**
Median age (range)	**32 (6-70)**	**53 (36-71)**
Patient diagnosis at transplantation		
Acute myeloblastic leukemia (AML)	**7**	**0**
Acute promyeloblastic leukemia (APL)	**3**	**0**
Acute lymphoblastic leukemia (ALL)	**6**	**0**
Chronic myeloblastic leukemia (CML)	**2**	**0**
Diffuse large B cell lymphoma (DLBC)	**1**	**12**
Follicular cell lymphoma (FCL)	**0**	**5**
Aplastic anemia (AA)	**1**	**0**
Myelodysplastic syndrome (MDS)	**5**	**0**
Multiple myeloma (MM)	**0**	**7**
Nonhematologic cancer	**1**	**2**
Conditioning regimen		
TBI-containing	**11**	**4**
CY/TBI	**6**	**0**
Flu/L-PAM/TBI	**5**	**0**
L-PAM/TBI	**0**	**4**
Non-TBI-conditioning	**15**	**22**
Flu	**4**	**0**
Flu/Bu	**5**	**0**
Flu/L-PAM	**6**	**0**
VP-16/CY	**0**	**5**
MCNU/IFO/CBDCA/VP-16	**0**	**10**
MCNU/L-PAM/Ara C/VP-16	**0**	**7**

TBI indicates total body irradiation; CY, cyclophosphamide; Flu, fludarabine; Bu, busulfan; L-PAM, melphalan; VP-16, etoposide; MCNU, ranimustine; IFO, ifosfamide; CBDCA, carboplatin; Ara C, cytarabine.

Cytokine Evaluation

Blood samples from patients were collected into tubes containing sodium citrate or tubes without any anticoagulant and the blood was allowed to clot at room temperature for a minimum of 1 hour. Then serum or citrated plasma was isolated by centrifugation for 20 minutes at 1000 × *g* at 4°C. The serum was divided into aliquots and frozen at −30°C until use. As a positive control, recombinant products were used in each assay, as well as standard solutions provided with the commercial kits. Human tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-4, and IL-6 ELISA kits were purchased from BioSource International, Inc. (Camarillo, CA). Serum levels of cytokines were measured according to the manufacturer's instructions. Normal ranges were as follows: TNF-α: 5-20 pg/mL, IFN-γ: 0-12.5 pg/mL, IL-4: 0-3.5 pg/mL, and IL-6: 0.2-4.5 pg/mL.

Measurement of Soluble (s)FasL, sIL-2 Receptor (R), sVascular Cell Adhesion Molecule (VCAM)-1, sE-Selectin, Ang-1, and Ang-2

sFasL, sIL-2R, sVCAM-1, sE-selectin, Ang-1, and Ang-2 ELISA kits were purchased from

Table 2. *Allogeneic SCT Outcome*

	Value
Donor sourse	
Related	7
HLA-identical sibling	4
Mismatched family member	2
HLA-matched family member	1
Unrelated	19
HLA-matched	12
Mismatched	7
Stem cell sourse	
Bone marrow transplantation (BMT)	6
Peripheral blood stem cell transplantation (PBSCT)	12
Cord blood transplantation (CBT)	8
Alive/dead	16/10
Cause of death progression	2
Death without progression	6
GVHD	1
Infection	3
TMA	2
Not described	2
GVHD	
Acute GVHD	26
Chronic GVHD	7

GVHD indicates graft-versus-host disease; TMA, thrombotic microangiopathy.

BioSource International Inc. For measurement of sFasL, sIL-2R, sVCAM-1, and sE-selectin in serum, all the kits were used according to the manufacturer's instructions. Normal ranges were as follows: sFasL: 0.02-0.14 ng/mL, sIL-2R: 150-450 IU/mL, sVCAM-1: 395-714 ng/mL, sE-selectin: 23.0-79.2 ng/mL, Ang-1: 1.8-13.2 ng/mL, and Ang-2: 1.5-4.6 ng/mL.

Activation of Endothelial Cells

Endothelial cells isolated from freshly obtained human umbilical cord veins were cultured according to the method of Jaffe and colleagues [36]. Second-passage cells were grown to confluence in 25 cm^2 culture flasks (3-4 days). The cells were subcultured in 12-well plates containing M-199 with growth supplement, fetal bovine serum, and heparin, after which they were washed once with 10 mM EDTA in phosphate-buffered saline to remove calcium-dependent binding proteins such as vitamin K-dependent coagulation factors. EDTA was subsequently removed by 3 washes with 0.4% fetal bovine serum albumin. Then, the cells were incubated at 37°C with 10 ng/mL of TNF-α (BioSource International Inc.), 250 ng/mL of Ang-1 or Ang-2 (R&D Systems, Minneapolis, MN) in M-199, 0.4% BSA. Samples of the cells containing FITC-labeled anti-annexin V or PE-labeled CD51 were added to Falcon tubes and analyzed using the Ortho Cytoron Absolute Analyzer.

Assessment of Endothelial Cell-Derived Microparticles (EDMPs)

EDMPs were detected using a previously reported method with some modifications [37]. A 10-μL aliquot of washed intact platelets (3×10^8/mL) was added to the plasma, and the mixture was incubated for 30 minutes in dark at room temperature, with FITC-labeled Annexin V (FITC-Ann V) and phycoerythrin (PE)-labeled CD51 (αvβ3) to detect EDMP. The samples were diluted 1:10 with HEPES-Tyrode's buffer containing 5 mmol/L EGTA and analyzed using the Ortho Cytoron Absolute Analyzer (Ortho Diagnostic Systems, Inc., Tokyo, Japan), set to detect only the particles bound to FITC-labeled Annexin V and PE-labeled CD51. This method was designed to ensure the detection of only procoagulant EDMP. The concentrations of these microparticles were then calculated per microliter of the whole blood.

Statistical Analysis

Results are shown as the mean ± standard errors. Variables in the 2 distribution groups were compared using the Mann-Whitney *U*-test or *t*-tests, as appropriate. Statistical significance was defined as a 2-tailed value of $P < .05$. The correlation between Ang-2 and other continuous-response variables was assessed by univariate and multivariate linear regression analysis. The disease-free survival (DFS) was calculated from the date of transplant to the date of relapse or any cause of death. The survival curves were estimated using the method of Kaplan and Meier.

RESULTS

SCT-Related Complications

Twenty-six patients who received allogeneic SCT developed aGVHD (grade I, 11; grade II, 9; grade III, 4; grade IV, 2) (Table 2). Three patients (grade III and IV) who received allogeneic SCT had severe complications and died within the first 4 weeks. Another, who had received autologous SCT, suffered from severe sepsis and died early. Therefore, 4 patients—3 from those who received allogeneic SCT and 1 who received autologous SCT—were excluded from the analysis of the present study.

Changes in Serum Cytokines and Soluble Factors

The level of cytokines and soluble factors before and after SCT is shown in Table 3. IFN-γ levels were found to be significantly higher 3 weeks after allogeneic SCT than beforehand, whereas IL-4 and IFN-γ levels after autologous SCT remained almost unchanged. In contrast, TNF-α, IL-6, sIL-2R, sVCAM-1, and sE-selectin exhibited a significant elevation after both autologous and allogeneic SCT, although the changes after autologous SCT were temporary. Levels of sIL-2R, sVCAM-1, and sE-selectin

Table 3. *Changes of Cytokines and Soluble Factors with SCT*

Cytokine/Factor	Before SCT	After SCT			
		1 Week	2 Weeks	3 Weeks	4 Weeks
Allo SCT (n = 23)[a]					
IFN-γ (pg/mL)	9.2 ± 0.5	11.8 ± 0.8	13.4 ± 0.5	14.5 ± 1.2*	14.9 ± 0.9**
IL-4 (pg/mL)	5.4 ± 0.4	5.4 ± 0.7	5.8 ± 0.4	5.8 ± 0.5	5.6 ± 0.5
TNF-α (pg/mL)	12.4 ± 0.8	16.7 ± 1.3	24.5 ± 1.9*	28.5 ± 5.7**	23.9 ± 1.6*
IL-6 (pg/mL)	21.3 ± 6.2	34.7 ± 6.5	51.8 ± 14.2**	49.7 ± 10.1*	47.6 ± 15.1*
sIL-2R (IU/mL)	725 ± 61	757 ± 73	1,033 ± 69*	1,134 ± 52*	1,337 ± 98**
sVCAM-1 (pg/mL)	965 ± 66	1,139 ± 73	1,366 ± 91*	1,579 ± 91**	1,631 ± 123**
sE-selectin (ng/mL)	66.5 ± 3.8	72.2 ± 4.4	90.5 ± 4.7*	118.5 ± 5.5**	128.8 ± 7.2**
Auto SCT (n = 25)[b]					
IFN-γ (pg/mL)	6.4 ± 0.8	6.2 ± 0.7	5.9 ± 0.6	5.8 ± 0.6	6.4 ± 0.8
IL-4 (pg/mL)	3.6 ± 0.5	3.7 ± 0.7	3.5 ± 0.8	3.1 ± 0.7	2.9 ± 0.9
TNF-α (pg/mL)	8.6 ± 1.1	10.2 ± 1.4	14.8 ± 1.9*	10.6 ± 1.2	9.8 ± 0.9
IL-6 (pg/mL)	12.5 ± 2.3	38.8 ± 22.1*	42.8 ± 12.5*	19.8 ± 5.2	15.6 ± 4.4
sIL-2R (IU/mL)	853 ± 148	1,458 ± 361*	1,429 ± 154*	1,170 ± 165	1,039 ± 113
sVCAM-1 (pg/mL)	1217 ± 86	1,165 ± 122	1,449 ± 65*	1,252 ± 97	1,128 ± 120
sE-selectin (ng/mL)	84.6 ± 10.3	79.7 ± 4.6	91.7 ± 10.2*	119.5 ± 13.1*	94.8 ± 9.3

TNF-α indicates tumor necrosis factor α; IFN-γ, interferon γ; IL-4, interleukin 4; IL-6, interleukin 6; sIL-2R, soluble interleukin-2 receptor; sVCAM-1, soluble vascular cell adhesion molecule-1; sE-selectin, soluble E-selectin.

*$P < .05$; **$P < .01$.

[a]Three patients who had severe complications and died after the procedure were excluded from the analysis of the present study (see "SCT-related complications in Results."

[b]One patient who suffered from severe sepeis was excluded from the analysis of the present study (see "SCT-related complications in Results," Data represent means ± standard error.

after allogeneic SCT continued to increase for up to 4 weeks.

Changes in sFasL, EDMP, Ang-1, and Ang-2

Figure 1 shows the changes in sFasL, EDMP, Ang-1, and Ang-2 levels after SCT. The level of sFasL in the group that received allogeneic SCT peaked within 3 weeks (0.32 ± 0.05 ng/mL, $P < .001$) and then decreased. The level of EDMP showed the same tendency as sFasL (allogeneic SCT: 2 weeks, 491 ± 53/μL, $P < .05$; 3 weeks, 595 ± 87/μL, $P < .01$, 4 weeks, 522 ± 59/μL, $P < .05$). The level of Ang-2 in allogeneic SCT continued to increase for up to 4 weeks (2 weeks, 3.9 ± 0.5 ng/mL, $P < .05$; 3 weeks, 4.7 ± 0.4 ng/mL, $P < 0.05$; 4 weeks, 5.8 ± 0.7 ng/mL, $P < .01$), although the level of Ang-1 did not show significant changes. In contrast, levele of sFasL, EDMP, Ang-1, and Ang-2 in the recipients of autologous SCT did not show significant changes. There were no significant correlations between sFasL, EDMP, Ang-1, and Ang-2 in the recipients of allogeneic SCT.

Figure 2 shows the changes in levels of sFasL and EDMP at 4 weeks in the recipients of allogeneic SCT with elevated Ang-2 levels. sFas and EDMP levels at 4 weeks in allogeneic SCT with elevated Ang-2 (greater than the mean + 2 standard deviations of basal levels) were used for the analysis. Ten patients had high Ang-2 levels (Ang-2 >4.5 ng/mL). The patients with high Ang-2 exhibited significant increase of sFasL and EDMP compared with those without high Ang-2.

GVHD, DFS, and Angiopoietins in Patients after Allogeneic SCT

The actual incidence of high-grade aGVHD (grade II-IV) was 52.8%. Projected DFS at 1000 days with and without high-grade aGVHD was 0.37 ± 0.04 versus 0.78 ± 0.06 ($P = .04$; Figure 3). Figure 4 shows the changes in levels of Ang-2 at 4 weeks with and without high-grade aGVHD in the recipients of allogeneic SCT. The patients with high-grade GVHD exhibited significant increases in Ang-2 compared to patients with low-grade GVHD. However, the DFS at 1000 days exhibited no significant differences (Figure 5). Table 4 shows the relationship between Ang-2 and cytokines/soluble factors in univariate and multivariate regression analysis. Simple linear regression analysis showed significant correlations that were performed on TNF-α, IL-6, sIL-2R, sVCAM-1, sE-selectin, sFasL, and EDMP. We logarithmically transformed and calculated all variables. Before multiple linear regression analysis, we performed Pearson's product-moment correlation analysis among all relevant variables. In cases where the correlation coefficients (r) exceeded 0.8 in these variables, we selected the ones that presented with the higher β regression coefficient by simple linear regression analysis. In the multiple linear regression model, IFN-γ, TNF-α, sVCAM-1, sFasL, and EDMP levels

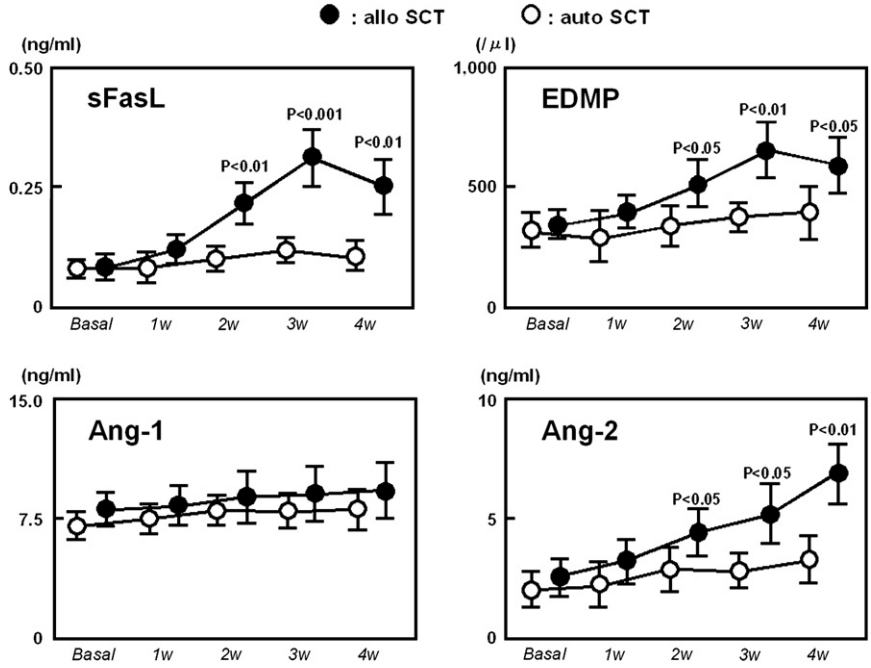

Figure 1. Changes of sFasL, EDMP, Ang-1, and Ang-2 in patients undergoing allogeneic and autologous SCT. Blood samples were obtained on days 0 (basal), 7 (1 weeks), 14 (2 weeks), 21 (3 weeks), and 28 (4 weeks) after the transplantation. Error bars show standard error. Student's *t*-test was used for statistical comparisons.

were investigated. EDMP and sFas-L were significantly correlated with Ang-2 (Table 4).

Relationship between Ang-1, Ang-2, and EDMP in Activated Endothelial Cells

Figure 6 shows the release of EDMP from endothelial cells caused by TNF-α and detected by flow cytometry using FITC-labeled Annexin V and PE-labeled CD51 (Figure 6A and B). TNF-α promoted the release of EDMP (Figure 6C) and Ang-1 inhibited this promotion (Figure 6D). However, this effect of Ang-1 was partially inhibited by the addition of Ang-2 (Figure 6E).

DISCUSSION

SCT provides an opportunity to trace the process of hematopoietic reconstitution in vivo, and many

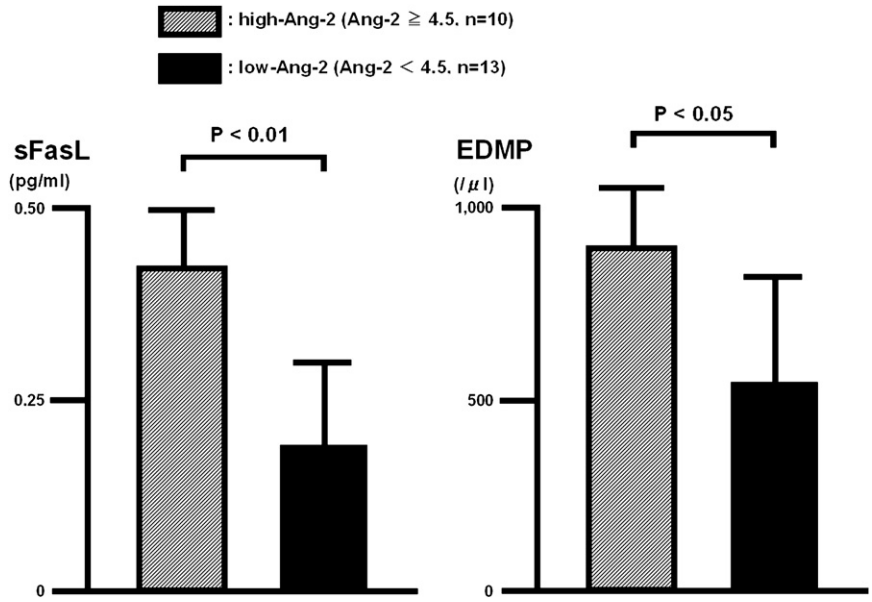

Figure 2. sFasL and EDMP levels in patients after allogeneic SCT with and without high-Ang-2. Values presented as mean ± standard error.

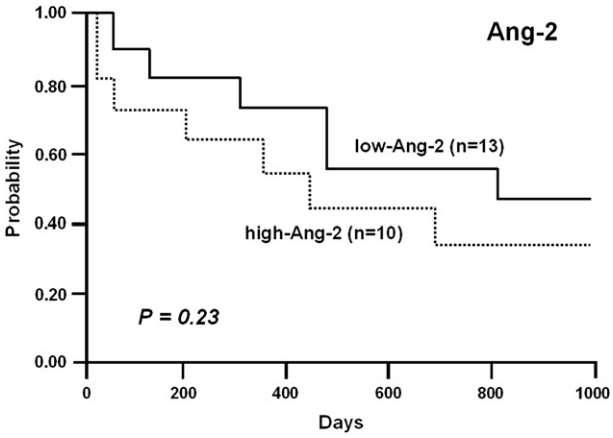

Figure 3. Probability of DFS in aGVHD. The estimated probability of DFS at 2 years was 78% (grade I) and 37% (grade II-IV).

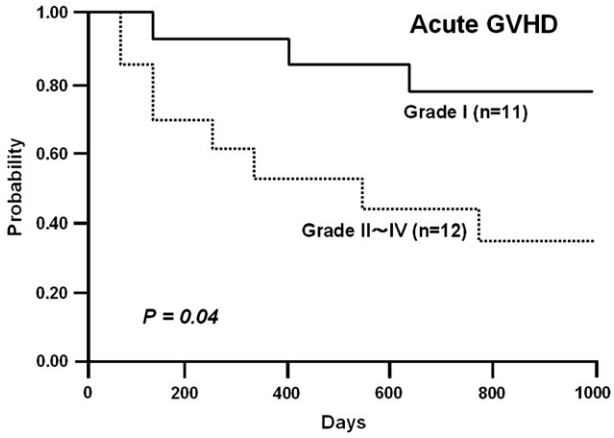

Figure 5. Probability of DFS in relation to Ang-2 levels. The estimated probability of DFS at 2 years was 56% (low-Ang-2) and 44% (high-Ang-2).

cytokines are known to control the process of hematopoiesis. In the present study, several cytokines and soluble factors were measured. TNF-α, IL-6, sIL-2R, sVCAM-1, and sE-selectin exhibited a significant elevation after both autologous and allogeneic SCT, although the changes after autologous SCT were temporary. In addition, sFasL and EDMP were measured before SCT and serially after SCT, and these factors exhibited a significant elevation in the early phase (2 or 3 weeks) after SCT (Figure 1). It has previously been reported that certain cytokines and soluble factors are useful for the diagnosis of GVHD after allogeneic SCT [38-40]. Proinflammatory cytokines including IFNγ, IL-6, and TNF-α are important mediators and regulators of GVHD [38,39]. sIL-2R appears to be a convenient marker for the detection of aGVHD [40]. In the present study, the involvement of sFasL in the elevation of IL-6, TNF-α, and sIL-2R after allogeneic SCT appeared to be important, because sFasL exhibited the same changes as these cytokines and soluble factors [41-43]. EDMP also exhibited changes similar to those of sFasL. It is reported that EDMP exhibit a dynamic change after SCT [16]. In particular, the increase of EDMP in the TMA/thrombotic thrombocytopenic purpura (TTP) case was quite remarkable [16,44]. Recently, Pihusch et al. [45] reported that elevation of EDMPs during aGVHD might induce endothelial cell injury after hematopoietic SCT and might serve as a diagnostic test for early differentiation of aGVHD from other transplant-related complications. Thus, our results suggest the possibility that sFasL and EDMP play a role in GVHD after allogeneic SCT [11-13,46].

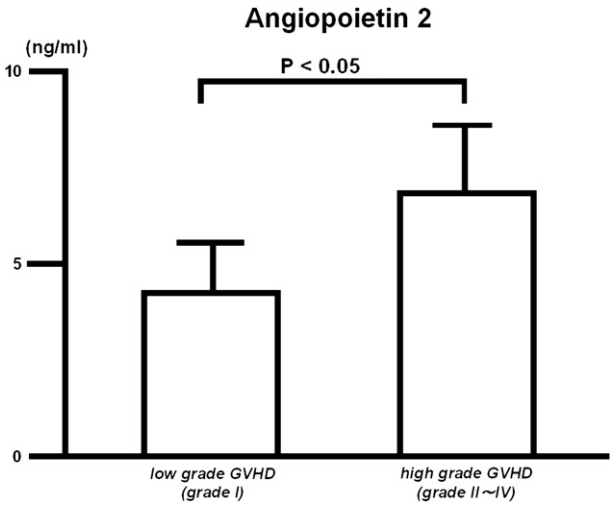

Figure 4. Ang-2 levels in patients after allogeneic SCT with and without high-grade GVHD. Values presented as mean ± standard error.

Table 4. *Relationship between Ang-2 and Cytokines/Soluble Factors in Multivariate Regression Analysis*

	Univariate Analysis		Multivariate Analysis
Variable	***R***	***P* value**	***P* value**
IFN-γ (pg/mL)	**0.038**	**.456**	**.634**
IL-4 (pg/mL)	**0.066**	**.293**	
TNF-α (pg/mL)	**0.237**	**<.05**	**.419**
IL-6 (pg/mL)	**0.259**	**<.05**	
sIL-2R (IU/mL)	**0.218**	**<.05**	
sVCAM-1 (pg/mL)	**0.297**	**<.01**	**.184**
sE-selectin (ng/mL)	**0.231**	**<.05**	
sFasL (pg/mL)	**0.312**	**<.01**	**<.05**
EDMP (/μL)	**0.358**	**<.001**	**<.01**
Ang-1 (ng/mL)	**0.091**	**.552**	

IFN-γ indicates interferon γ; IL-4, interleukin 4; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; sIL-2R, soluble interleukin-2 receptor; sVCAM-1, soluble vascular cell adhesion molecule-1; sE-selectin, soluble E-selectin; sFasL, soluble Fas ligand; EDMP, endothelial cell-derived microparticle; Ang-1, angiopoietin-1.

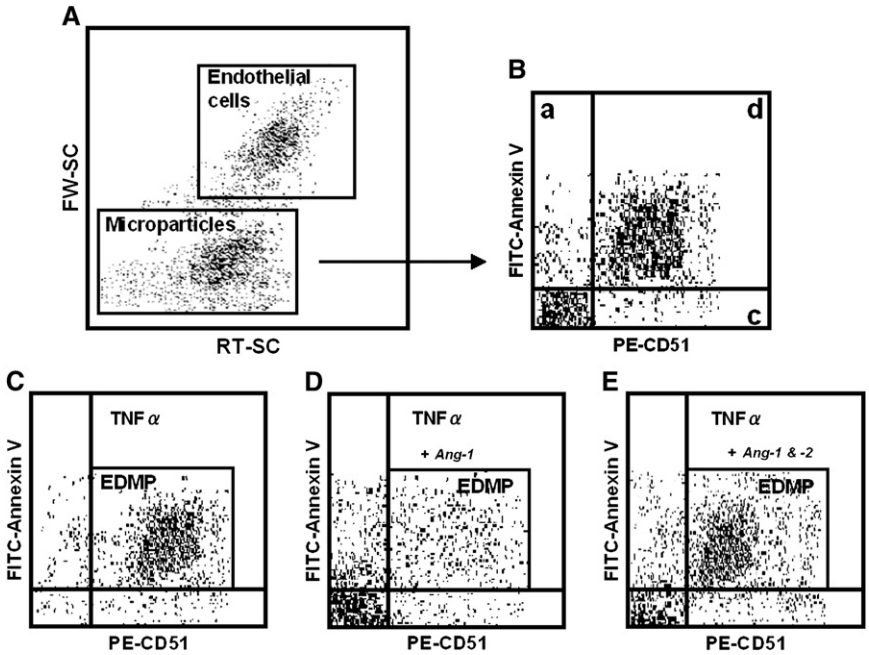

Figure 6. Flow cytometric analysis of EDMP. Only particles positive for FITC-labeled Annexin V and PE-CD51 were gated to distinguished endothelial cells and EDMP from electric noise (A, B). EDMPs were elevated by stimulation of TNF-α (C), and this elevation was inhibited the addition of Ang-1 (D). Furthermore, the suppressive effect of Ang-1 was partially inhibited by the addition of Ang-2 (E).

Although many cytokines are known to control the process of hematopoiesis after SCT, the role of angiopoietins in this process is not well understood. In the present study, the level of Ang-2 in allogeneic SCT continued to increase for up to 4 weeks, although the level of Ang-1 did not show significant changes. In addition, the patients with high Ang-2 exhibited significant increase of sFasL and EDMP compared with those without high Ang-2 (Figure 2). To understand the relationship between Ang-2 and GVHD, we analyzed the DFS. In the present study, the actual incidence of high-grade aGVHD (grade II-IV) was 52.8%, and projected the DFS at 1000 days with and without high-grade aGVHD was 0.37 ± 0.04 and 0.78 ± 0.06, respectively. In addition, the patients with high-grade GVHD exhibited a significant increase in Ang-2 compared to patients with low-grade GVHD. Thus, our results suggest the promotional effect of Ang-2 on GVHD, although the actual DFS at 1000 days with or without high Ang-2 exhibited no significant differences. Ang-1, a major physiological ligand for Tie2 receptor, is responsible for the recruitment and stable attachment of pericytes resulting in vascular maturation in the process of angiogenesis [24]. Ang-1 has been shown to promote endothelial cell survival without causing proliferation [47], and to stabilize endothelial interactions with surronding support cells [27]. It can block the vascular permeability effects of VEGF in vivo [27,28]. In addition, the VEGF-induced vascular permeability appears to be a cause of capillary leak syndrome, which is 1 of the transplant-related complications. We previously reported that platelet activation markers such as sCD40L, platelet-derived MP and RANTES continued to increase after allogeneic SCT [48]. In addition, activated platelets release Ang-1 [49-51].

On the other hand, Ang-2 competitively inhibits the binding of Ang-1 to Tie2, acting like a natural antagonist and rendering blood vessels "unstable" [52]. Also, it is reported that Ang-2 is rapidly released upon stimulation from endothelial cell [53]. When endothelial cells are activated after SCT, they could generate Ang-2. In this manner, Ang-2 generation after allogeneic SCT makes it possible to promote GVHD because of the inhibition of Ang-1-related function. We performed the in vitro experiment using endothelial cells to understand whether Ang-2 plays a pathogenic role in the endothelial injury, which occurs during aGVHD. Ang-1 inhibited the release of EDMP promoted by TNF-α, and this effect of Ang-1 was partially inhibited by the addition of Ang-2. These results suggest that the increase in Ang-2 promotes the endothelial injury related to GVHD. Indeed, in the present study, the multivariate regression analysis showed that EDMP and sFasL were significant factors in the elevation of Ang-2. However, our study has some limitations: it was a small sample size, and some patients who had severe complications were excluded from the analysis of the present study. Therefore, further examination will be necessary to establish the exact mechanism of Ang-2-related promotion for GVHD after allogeneic SCT.

In conclusion, we measured and compared levels of cytokines and soluble factors in patients undergoing SCT. Ang-2, sFasL, and EDMP exhibited particular changes after SCT. Our results suggest that Ang-2 generation after allogeneic SCT relates to the promotion of GVHD.

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