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ORIGINAL ARTICLE

Circulating Levels of Adipokines Predict the Occurrence of Acute Graft-versus-host Disease

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Currently, detecting biochemical differences before and after allogeneic stem cell transplantation (SCT) for improved prediction of acute graft-versus-host disease (aGVHD) is a major clinical challenge. In this pilot study, we analyzed the kinetics of circulating adipokine levels in patients with or without aGVHD before and after allogeneic SCT. Serum samples were obtained and stored at −80°C within 3 hours after collection, prior to conditioning and at engraftment after transplantation. A protein array system was used to measure the levels of 7 adipokines of patients with aGVHD (n=20) and without aGVHD (n=20). The resistin level at engraftment was significantly increased ($p<0.001$) after transplantation, regardless of aGVHD occurrence. In the non-aGVHD group, the concentrations of the hepatocyte growth factor (HGF) (mean values±SD; 206.6±34.3 vs. 432.3±108.9 pg/ml, $p=0.040$) and angiopoietin-2 (ANG-2) (mean values±SD; 3,197.2±328.3 vs. 4,471.8±568.4 pg/ml, $p=0.037$) at engraftment were significantly higher than those of the pre-transplant period, whereas in the aGVHD group, the levels of adipokines did not change after transplantation. Our study suggests that changes in serum HGF and ANG-2 levels could be considered helpful markers for the subsequent occurrence of aGVHD.

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Keywords: Adipokines, Acute graft-versus-host disease, Allogeneic stem cell transplantation

INTRODUCTION

Acute graft-versus-host disease (aGVHD) is a major barrier to the successful application of allogeneic stem cell transplantation (SCT). Donor T cells that recognize minor or major histocompatibility differences in the host are critical mediators of aGVHD (1). Numerous trials have been conducted to determine the role of T-cell subsets in clinical aGVHD by studying specific cytokines in the serum of patients with GVHD (2-4) and in lesional tissue biopsies (5-7). Recently, investigators have postulated the development of biomarkers from other non-human leukocyte antigen (HLA)-dependent factors, such as polymorphisms of cytokine genes and gene expression profiles of $CD4^+$ and $CD8^+$ T cells from donors, as predictors of aGVHD.

White adipose tissue is no longer considered an inert tissue mainly devoted to energy storage, rather an active participant in regulating physiologic and pathologic processes including immunity and inflammation. Furthermore, cross-talk between lymphocytes and adipocytes can lead to immune regulation. Adipose tissue produces and releases a variety of proinflammatory and anti-inflammatory factors including adipokines, cytokines, and chemokines. Altered adipokine levels have been observed in a variety of inflammatory conditions. Hence, proinflammatory molecules produced by adipose tissue may be implicated as active participants in the develop-

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Abbreviations: aGVHD, acute graft-versus-host disease; AML, acute myeloid leukemia; ANG-2, angiopoietin-2; ATG, anti-thymocyte globulin; CRP, C-reactive protein; HGF, hepatocyte growth factor; MDS, myelodysplastic syndrome; PAI-1, plasminogen activator inhibitor-1; VEGF, vascular endothelial growth factor

ment of aGVHD after allogeneic SCT (8).

Whether adipokines are associated with a high risk of aGVHD development in patients after allogeneic SCT remains to be well understood. The purpose of this study was to determine whether there is greater adipokine production in the sera of aGVHD patients than in the sera of non-aGVHD patients. We tested the hypothesis that the change in circulating levels of each adipokine from prior to conditioning therapy through engraftment would predict the occurrence of aGVHD. We compared serum levels of each adipokine in paired samples from aGVHD and non-aGVHD patients.

MATERIALS AND METHODS

Patients and transplant procedures

Our primary aim was to investigate an association between adipokines and the occurrence of aGVHD. This study was approved by the Institutional Review Board and conducted in accordance with the Declaration of Helsinki. This study examined 40 adult patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) who underwent allogeneic SCT at our institute between February 2013 and February 2014. Twenty patients who developed grade 2～4 aGVHD (14 with grade 2 and 6 with grade 3～4) and twenty patients who never developed aGVHD were included in the analyses of adipokines.

Donor selection was based on molecular typing for HLA-A, HLA-B, HLA-C, and DRB1. Patients received either a myeloablative conditioning regimen (n=16), including total body irradiation (TBI)/cyclophosphamide or busulfan/cyclophosphamide, or a reduced-intensity regimen (n=24), including fludarabine and busulfan with TBI 400 cGy. Overall, four patients received bone marrow, and 36 received granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSCs). Antithymocyte globulin (ATG, Genzyme, Cambridge, MA, USA; 2.5 mg/kg) was administered as a part of the conditioning regimen to reduce aGVHD in patients who received not only transplants from mismatched unrelated donors but also PBSCs from matched unrelated donors. GVHD prophylaxis was attempted by administering a calcineurin inhibitor (cyclosporine for the majority of related transplants and tacrolimus for all unrelated transplants) along with short-term administration of methotrexate. The calcineurin inhibitor dose was tapered gradually starting on day 100 to day 120 after allogeneic SCT in the absence of aGVHD. The other general transplantation procedures were performed as described previously (9,10).

Sample preparation

Serum samples for the analyses of adipokines were taken before the start of the conditioning regimen and after the neutrophil engraftment, which was defined as the first of three consecutive days with an absolute neutrophil count $>0.5\times10^9$/L.

Luminex magnetic screening assays

Interleukin-8 (IL-8), angiopoietin-2 (ANG-2), plasminogen activator inhibitor-1 (PAI-1), resistin, intercellular adhesion molecule-1 (ICAM-1), C-reactive protein (CRP), and hepatocyte growth factor (HGF) levels were tested using a commercially available Luminex Magnetic Screening Assay (R&D Systems, Minneapolis, MN, USA). Each sample was tested in duplicate, and the average is reported in picograms per milliliter. The sensitivity of detection ranged from 15.9 to 3,863 pg/ml for IL-8, 120 to 29,175 pg/ml for ANG-2, 22.6 to 5,500 pg/ml for PAI-1, 44.7 to 10,870 pg/ml for resistin, 3,011 to 731,629 pg/ml for ICAM-1, 141 to 34,174 pg/ml for CRP, and 33.6 to 8,166 pg/ml for HGF.

Statistical analysis

Statistical comparisons between groups were performed using a χ^2 or Fisher's exact test for categorical variables or the non-parametric Mann-Whitney U test for continuous variables. Mean values of adipokines obtained from ELISA assays performed on pre-SCT and post-SCT samples were compared with the non-parametric Mann-Whitney U test for continuous variables. Next, the change in each adipokine at engraftment relative to before the conditioning regimen was initiated was compared using a paired t-test in GVHD and no GVHD groups.

RESULTS

Demographic characteristics

The clinical characteristics of patients are shown in Table I. Forty patients were included in the non-aGVHD (n=20) and aGVHD (n=20) groups consisting of 23 males and 17 females with a median age of 47.5 years (range, 21～64). The patients were diagnosed as AML (n=29) and MDS (n=11), and received transplants from sibling donors (n=16), unrelated donors (n=8), or HLA-mismatched familial donors (n=16). At the time of the transplant, 14 patients (35%) had advanced disease features, which are defined as AML beyond the first remission and high-risk MDS (international prognostic scoring

Table I. Patients and transplantation characteristics

Parameters	No aGVHD (n=20) (%)	aGVHD (≥grade 2) (n=20) (%)	p
Age (y), median (range)	46.5 (24~61)	48 (21~64)	ns
Sex of patient, M/F	12 (60)/8 (40)	11 (55)/9 (45)	ns
Sex of donor, M/F	10 (50)/10 (50)	11 (55)/9 (45)	ns
Sex pair			ns
Female to male/Others	7 (35)/13 (65)	6 (30)/14 (70)	
Diagnosis			ns
AML/MDS	16 (80)/4 (20)	13 (65)/7 (35)	
Pre-SCT disease status			ns
Standard/Advanced	14 (70)/6 (30)	12 (60)/8 (40)	
Donor type			ns
Sibling/Unrelated/FMT	9 (45)/3 (15)/8 (40)	7 (35)/5 (25)/8 (40)	
Sources of graft			ns
BM/PBSC	2 (10)/18 (90)	2 (10)/18 (90)	
Conditioning regimen			ns
TBI based/non-TBI based	14 (70)/6 (30)	16 (80)/4 (20)	
Conditioning intensity			ns
MAC/RIC	7 (35)/13 (65)	9 (45)/11 (55)	
ATG containing			ns
Yes/No	14 (70)/6 (30)	15 (75)/5 (25)	
GVHD prophylaxis			ns
CS based/FK506 based	9 (45)/11 (55)	7 (35)/13 (65)	

aGVHD indicates acute graft-versus-host disease; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; BM, bone marrow; CS, cyclosporine; F, female; FMT, HLA-mismatched familial donor; GVHD, graft-versus-host disease; M, male; MAC, myeloablative conditioning; PBSC, peripheral blood stem cell; y, year-old; RIC, reduced-intensity conditioning; TBI, total body irradiation

system ≥intermediate-2). There were no significant differences between the non-aGVHD and aGVHD groups for median age, sex, pre-SCT disease status, donor type, sources of graft, conditioning intensity, and GVHD prophylaxis.

Comparison of the serum levels of each adipokine between pre-SCT and post-SCT (at engraftment)

We compared each adipokine level at engraftment with those before the start of the conditioning regimen in all the patients. Resistin levels at engraftment were significantly higher ($p<0.001$) than those of pre-SCT, and HGF levels at engraftment showed a tendency to be higher ($p=0.071$) than those before SCT (Table II).

Relationship between the changes of each adipokine level and occurrence of aGVHD

Next, we analyzed the change of each adipokine level from pre-SCT to post-SCT in the two groups. In the non-aGVHD group, the pre- and post-SCT HGF levels were 206.6±34.3 and 432.3±108.9 pg/ml, respectively, ($p=0.0404$, Fig. 1A) and the pre- and post-SCT ANG-2 levels were 3,197.2±328.3 and 4,471.8±568.4 pg/ml, respectively, ($p=0.0366$, Fig. 1B). On the contrary, in the aGVHD group, the levels of HGF and ANG-2 were not significantly different before and after SCT. Resistin levels at engraftment were significantly higher than those of pre-SCT in both groups (non-aGVHD and aGVHD group, $p<0.001$ and $p=0.0423$, respectively) (Fig. 1C). No significant changes in other parameters (IL-8, ICAM-1, PAI-1, and CRP) between pre-SCT and post-SCT groups were observed (Table III). Of note, PAI-1 levels analyzed prior to conditioning therapy were increased in the non-aGVHD cohort.

DISCUSSION

It is now well established that white adipose tissue functions as an active endocrine organ to modulate physiological metabolic processes. As adipose tissue contains various cell types such as adipocytes, immune cells, endothelial cells, and fibroblasts, it produces and releases diverse secretory proteins

Table II. The comparison of apokines between pre- and post-SCT

Parameters	Total patients (n=40)		
	Pre-SCT	Post-SCT (at engraftment)	p
IL-8, pg/ml	147.8±78.0	8.8±1.6	0.742
ICAM-1, pg/ml	$4.6\times10^5\pm0.8\times10^5$	$5.1\times10^5\pm0.7\times10^5$	0.149
HGF, pg/ml	263.4±51.3	392.5±75.8	0.071
ANG-2, pg/ml	3,374.0±362.3	4,231.3±460.8	0.132
PAI-1, pg/ml	$4.8\times10^4\pm0.5\times10^4$	$4.6\times10^4\pm0.4\times10^4$	0.686
Resistin, pg/ml	$1.4\times10^4\pm0.2\times10^4$	$3.0\times10^4\pm0.3\times10^4$	<0.001
CRP, pg/ml	$12.7\times10^4\pm2.5\times10^4$	$10.8\times10^4\pm1.9\times10^4$	0.245

aGVHD indicates acute graft-versus-host disease; SCT, stem cell transplantation. *t- test were utilized to compare the continuous variables (pre-SCT vs post-SCT). †Mean±SE

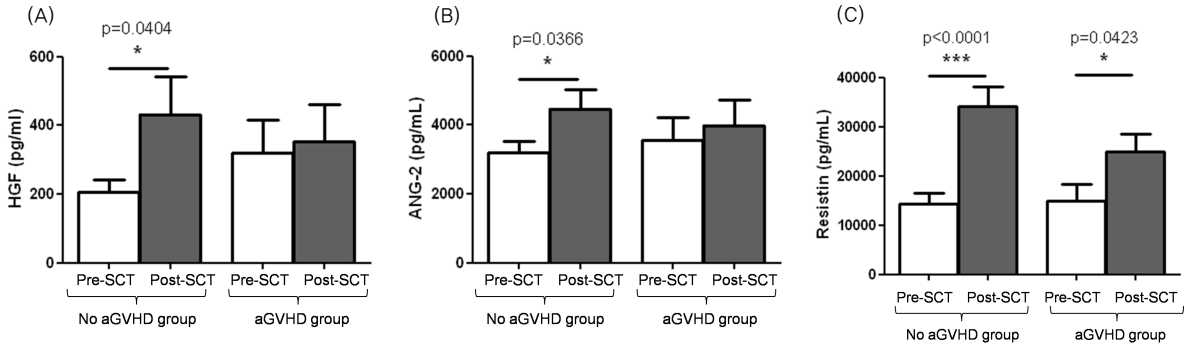

Figure 1. Relationship between changes in adipokines and occurrence of aGVHD. (A, B) Significant increasing of HGF (A) and ANG-2 (B) levels at engraftment relative to pre-SCT were observed in the no GVHD group but not in the aGVHD group. (C) Serum resistin levels were compared before and after allogeneic SCT according to the occurrence of GVHD. Abbreviations: HGF, hepatocyte growth factor; ANG-2, angiopoietin-2; SCT, stem cell transplantation; aGVHD, acute graft-versus-host disease.

Table III. The relation between changes of apokines and occurrence of aGVHD

Parameters	No aGVHD (n=20)			aGVHD (≥grade 2) (n=20)		
	Pre-SCT	Post-SCT (at engraftment)	p	Pre-SCT	Post-SCT (at engraftment)	p
IL-8, pg/ml	108.6±90.4	9.0±1.9	0.285	187.0±129.0	8.6±2.7	0.183
ICAM-1, pg/ml	$4.9\times10^5\pm1.1\times10^5$	$5.5\times10^5\pm1.2\times10^5$	0.130	$4.4\times10^5\pm1.2\times10^5$	$4.8\times10^5\pm1.0\times10^5$	0.278
HGF, pg/ml	206.6±34.3	432.3±108.9	0.040	320.3±96.4	352.7±107.6	0.743
ANG-2, pg/ml	3,197.2±328.3	4,471.8±568.4	0.037	3,550.9±654.1	3,990.8±736.4	0.177
PAI-1, pg/ml	$6.1\times10^4\pm0.7\times10^4$	$5.2\times10^4\pm0.7\times10^4$	0.144	$3.8\times10^4\pm0.5\times10^4$	$4.0\times10^4\pm0.3\times10^4$	0.568
Resistin, pg/ml	$1.4\times10^4\pm0.2\times10^4$	$3.4\times10^4\pm0.4\times10^4$	<0.001	$1.5\times10^4\pm0.3\times10^4$	$2.5\times10^4\pm0.4\times10^4$	0.042
CRP, pg/ml	$8.4\times10^4\pm1.5\times10^4$	$11.2\times10^4\pm0.3\times10^4$	0.312	$16.5\times10^4\pm4.5\times10^4$	$10.4\times10^4\pm2.7\times10^4$	0.072

aGVHD indicates acute graft-versus-host disease; SCT, stem cell transplantation. *Paired t- test were utilized to compare the continuous variables (pre-SCT vs post-SCT). †Mean±SE

called adipokines into the systemic circulation (11). In this study, we investigated whether pre- and post-transplant changes in serum levels of adipokines correlated with the subsequent development of aGVHD in patients undergoing allogeneic SCT and its potential use to anticipate the development of aGHD, allowing for early modification of immunosuppressive

therapy. HGF and ANG-2 levels pre- and post-SCT were significantly higher in the non-aGVHD group than in the aGVHD group, whereas resistin levels were significantly increased after transplantation, irrespective of the occurrence of aGVHD, and there was no statistical difference between the two groups.

Adipokines have been extensively studied for their involvement in obesity and associated morbidities, particularly cardiovascular disease, metabolic syndrome, and type 2 diabetes (12). Inflammation is the common thread generally invoked to regulate the production of adipokines in obesity and its comorbidities, and it can be involved in GVHD pathophysiology. Pathologically, in GVHD there is a modest inflammatory infiltrate but substantial damage to the basilar layer of the skin, the intestinal crypts, and the portal area of the liver. Thus, a reasonable framework in which to consider the induction of GVHD is that there is damage to the epithelium and endothelium by the conditioning regimen itself as well as by effects of the underlying disease. The injured tissues respond with the production of factors - cytokines, chemokines, and adhesion molecules, among others-that signal the immune system (13). In this study, a distinct aGVHD-related serum adipokine pattern was revealed using the luminex magnetic screening assay. The key adipokines that discriminated between aGVHD and non-aGVHD individuals were ANG-2 and HGF, suggesting that these two adipokines may have a protective effect against aGVHD.

Factors intrinsic to the endothelial cell system of the recipient may influence the outcome of allogeneic SCT. Recent studies have identified the occurrence of neovascularization during aGVHD (14), and several lines of evidence point to endothelial damage as a potential underlying cause of GVHD refractoriness (15,16). In this study, serum levels of ANG-2 showed no significant change over time in the cohort with aGVHD but were significantly higher at engraftment than before conditioning in the patients without aGVHD. We have shown that higher vascular endothelial growth factor (VEGF) levels have been associated with reduced severity of GVHD (9). It has been shown that *in vivo*, in the presence of endogenous vascular VEGF-A, ANG-2 promotes a rapid increase in capillary diameter, remodeling of the basal lamina, proliferation and migration of endothelial cells, and stimulates sprouting of new blood vessels (17). Our results are further substantiated by the finding that ANG-2/VEGF ratios may be used to anticipate the onset of GVHD because it has been demonstrated that ANG-2 mediates endothelial cell death if VEGF is concomitantly blocked.

HGF was originally identified and cloned as a potent mitogen for hepatocytes (18,19). It has been demonstrated that HGF gene transduction can reduce acute GVHD while preserving the graft-versus-tumor effects in a leukemia animal model (20). HGF exerts a potent protective effect on the thymus, which in turn promotes reconstitution of bone marrow-derived T cells after allogeneic SCT. Kuroiwa and colleagues were able to maintain continuous levels of the polyfunctional cytokine by repeatedly transfecting human HGF cDNA into skeletal muscle, and they observed that two of the characteristic findings in GVHD, crypt cell apoptosis and infiltration of lymphocytes into the portal triad, were absent in the treated animals (21). This study identified the protective effect of HGF although HGF levels were significantly associated with a higher probability of GVHD (22,23).

Resistin, a 10 kD-polypeptide with 114 amino acids in rodents, is identified as an inducer of pulmonary inflammation (24) and insulin resistance (25). Resistin is involved in the activation of SOCS3 resulting in the suppression of insulin-mediated signaling in adipocytes (26). Considering the crosstalk between inflammatory pathways and the insulin signaling cascade, resistin may represent a link between inflammation and metabolic signals (27). There is considerable controversy about the role of resistin in humans. In this cohort, serum resistin levels in patients undergoing allogeneic SCT were markedly higher at engraftment than before conditioning regardless of the occurrence of GVHD; this is in line with previous reports that resistin levels are associated with many inflammatory markers (28,29) and the severity of inflammatory diseases (30).

In summary, our study suggests that the changes in serum HGF and ANG-2 levels could be considered helpful markers for the subsequent occurrence of aGVHD with a potential GVHD protective effect. Upregulated HGF and ANG-2, occurring early after the SCT, may be associated with protection against aGVHD during allogeneic SCT, but further studies are needed. This is a preliminary study with a small number of patients. However, we assume that the effective utilization of the multiplex adipokine assay for the diagnosis of aGVHD and therapy design will allow for advanced disease management in the clinic. Further trials are needed focusing on the roles of adipokines in GVHD pathopysiology.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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