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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Diagnostic Value of Serum Procalcitonin and C-reactive Protein for Infections after Allogeneic Hematopoietic Stem Cell Transplantation versus Nontransplant Setting

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Abstract

Objective Procalcitonin (PCT) has been increasingly used as a biomarker of infection. The purpose of this study was to evaluate its diagnostic value after hematopoietic stem cell transplantation (HSCT), where non-infectious febrile complications such as graft-versus-host disease frequently develop.

Methods We retrospectively analyzed 144 febrile episodes (infections: 82, and noninfections: 62) in adult patients with hematological disorders, including 57 and 87 episodes in HSCT and non-HSCT patients, respectively.

Results Of 57 febrile episodes in HSCT patients, 46 (86%) and 25 (44%) revealed positivity for C-reactive protein (CRP) and PCT, respectively. Among 87 febrile episodes in non-HSCT patients, 81 (93%) and 22 (25%) events showed positive results of CRP and PCT. Both of these biomarkers were associated with infectious episodes in univariate analysis. Multivariate analysis showed that a high cut-off level (>9.5 mg/dL) of CRP was a better indicator for infections than PCT in HSCT patients, while PCT positivity was more diagnostic for infections than any cutoff CRP level in non-HSCT patients.

Conclusion It may be necessary to interpret the results of these biomarkers with different orders of priority in transplant versus nontransplant patients.

Key words: procalcitonin, C-reactive protein, infection, biomarker, stem cell transplantation

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Introduction

Infectious complications remain a major cause of morbidity and mortality following aggressive chemotherapy or hematopoietic stem cell transplantation (HSCT). Fever is the most important indicator of infections, but various noninfectious febrile episodes occur more frequently after allogeneic HSCT than after conventional chemotherapy. These include graft-versus-host disease (GVHD), engraftment syndrome (ES) (1), tumor fever, and reaction to drugs or blood products. In such immunocompromised patients, rapid distinction of infectious from noninfectious complications is important to provide immediate antibiotic treatment while avoiding unnecessary treatment that may cause drug toxicity and increased costs and bacterial resistance (2).

C-reactive protein (CRP) and procalcitonin (PCT) are convenient and helpful biomarkers of infections. The acute-phase protein CRP is produced by hepatocytes and its serum levels correlate well with the severity of inflammatory conditions (3). However, CRP positivity often lacks specificity for the detection of infections due to frequent elevation in various non-infectious complications (4, 5). PCT, the propeptide of calcitonin without hormonal activity, which was found as a novel biomarker of infection, has become increasingly popular and several studies have highlighted the usefulness of monitoring PCT levels for identifying infectious processes (6), guiding therapy (7), and predicting out-

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come (8). However, its usefulness remains controversial in HSCT recipients (9-12).

In this study, we retrospectively analyzed 144 febrile episodes that occurred in adult patients with hematological disorders and validated the diagnostic value of PCT and CRP for infectious complications in HSCT and non-HSCT patients.

Materials and Methods

Patients

Patients with hematological malignancies or aplastic anemia on anticancer chemotherapy or immunosuppressive therapy, who developed febrile episodes were included in this study. The medical records of 144 febrile episodes from 70 patients in whom the results of CRP and PCT measurements were available between December 2009 and July 2010 were reviewed. This study was approved by the Institutional Review Board of Kyushu University Hospital.

Classification of febrile episodes

Fever was defined as an axillary body temperature (BT) above 37.5℃, as used for the diagnostic criteria of febrile neutropenia (FN). The febrile episodes were classified into the following five groups: (i) Documented infection—bacterial infection if a pathogenic microorganism was isolated from a sterile sample or an opportunistic microorganism (e.g. commensal skin flora) was isolated from at least two consecutive blood cultures, fungal infection when clinical symptoms existed and fungi were isolated from sterile samples or invasive aspergillosis (IA) was confirmed according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group criteria (13), viral infection when clinical symptoms were noted and viral DNA was detected at the related organ or peripheral blood, and radiologically documented infection with typical clinical manifestations; (ii) FN—febrile episodes in patients whose absolute neutrophil count (ANC) was less than 0.5×10^9/L with negative cultures. (iii) Immune reactions—ES was characterized by fever, erythrodermatous skin rash, and noncardiogenic pulmonary edema during neutrophil recovery following HSCT (1), and GVHD was diagnosed according to well-established criteria (14); (iv) Drug—when drug administration was highly suspected as the cause of fever; (v) Others—when a noninfectious etiology including underlying disease, tumor lysis syndrome, or an episode that could not be classified in any of the previous groups. FN is not necessarily caused by infections, however major reason of FN patients not responding to empiric antibiotics is infection (e.g. fungus, virus, or bacteria resistant to therapy) (15). Thus, group (i) and group (ii) were classified as infectious episodes, while group (iii), group (iv), and group (v) were classified as noninfectious episodes.

Measurement and assessment of laboratory parameters

Blood samples to measure CRP and PCT were obtained within 24 hours after BT rose above 37.5℃. Serum levels of CRP were measured by a commercially available method, with a cut-off level of 0.5 mg/dL. Serum PCT concentrations were determined by a semi-quantitative immunochromatographic assay (Wako Junyaku, Osaka, Japan). The cut-off value was 0.5 ng/mL, and results were shown using the four-grade system as follows: negative [(-); <0.5 ng/mL], low-positive [(1+); <2 ng/mL], intermediate-positive [(2+); <10 ng/mL], and high-positive [(3+); ≥10 ng/mL] according to the manufacturer's recommendation.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of CRP and/or semiquantitative PCT were calculated on the basis of the clinical diagnosis of infectious episodes. We examined categorical variables, including sex, underlying diseases, treatment procedures, and serum PCT levels using the chi-square test. Numerical variables such as age, serum CRP concentration, and ANC were compared using Mann-Whitney's U-test. A p-value of <0.05 was considered statistically significant. All statistical analyses were conducted using the SPSS 17.0 program (SPSS Japan Inc., Tokyo, Japan). The optimal cutoff value of PCT and CRP was determined as the best possible combination of sensitivity and specificity (Youden index). The diagnostic reliability of these biomarkers for infections was evaluated by using receiver-operating characteristic (ROC) curve and the area under the ROC curve (AUC).

Results

Causes of febrile episodes

A total of 298 serum samples from 70 patients who reported a total of 144 febrile episodes were obtained to measure CRP and PCT levels. Patient characteristics are listed in Table 1. The median age of the patients was 54 years old, ranging from 19 to 78 years old. Fifty-seven episodes occurred after HSCT, 74 occurred after conventional chemotherapy, and 13 occurred during immunosuppressive treatment. Eighty-five episodes (59%) occurred in the neutropenic period defined as ANC $<0.5\times10^9$/L, while 59 (41%) occurred in nonneutropenic periods. Microbiologically or clinically documented infections were confirmed in 35 febrile episodes, including 12 gram-negative rod infections, 11 gram-positive cocci infections, two fungal infections, and one adenovirus infection. Although causative pathogens were not identified in the remaining 9 episodes, neutrophil accumulation with phagocytosis were documented by bronchoalveolar lavage fluid samples in 3 episodes with pneumonia, 4 episodes had enterocolitis without histological signs of gut GVHD, and 2 episodes had endotoxinemia.

Table 1. **Characteristics of the 144 Febrile Episodes**

Characteristics	Total	Procalcitonin		p value
		Positive (n = 47)	Negative (n = 97)	
Age, median (range)	54 (19-78)	54 (22-73)	54 (19-78)	NS
Sex (male/female)	74/70	20/27	54/43	NS
Underlying disease				NS
MDS/AML	51	15	36	
ALL	18	7	11	
ML	62	22	40	
Others*	13	3	10	
Treatment				0.02
Chemotherapy	74	17	57	
Stem cell transplantation	57	25	32	
Immunosuppressive therapy	13	5	8	
Procalcitonin level				NC
(-), < 0.5 ng/mL	97	0	97	
(1+), < 2.0 ng/mL	18	18	0	
(2+), < 10.0 ng/mL	16	16	0	
(3+), > 10.0 ng/mL	13	13	0	
CRP level (mg/dL), median (range)	3.90 (0.08-35.29)	6.37 (0.43-35.29)	3.01 (0.08-26.01)	< 0.0001
ANC (x10^9/L), median (range)	0.212 (0-27.054)	0.282 (0-27.054)	0.21 (0-10.392)	NS

MDS/AML indicates myelodysplastic syndrome/ acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma. *Others include multiple myeloma, aplastic anemia, primary myelofibrosis, and hemophagocytic syndrome. CRP, C-reactive protein; ANC, absolute neutrophil count; NS, not significant; NC, not calculated.

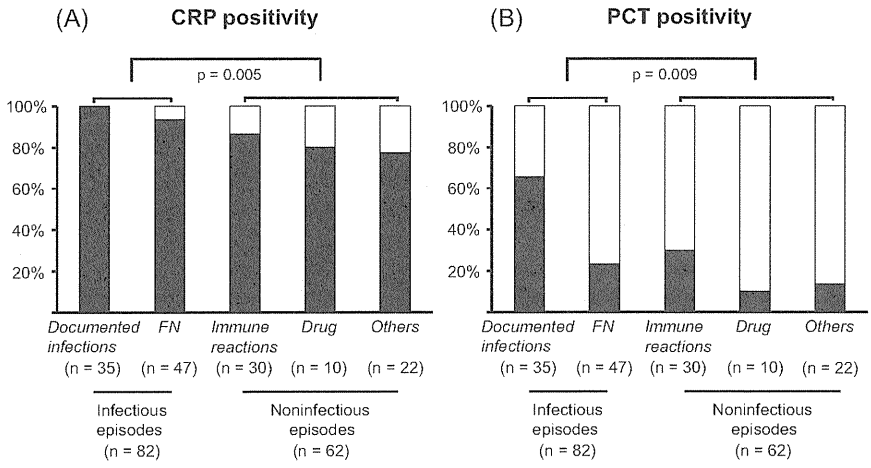

Figure 1. **Rates of (A) CRP and (B) PCT positivity according to the different etiologies of fever.**

Forty-seven episodes were categorized as FN, 30 as immune reactions, 10 as drug fever, and 22 as others.

CRP and PCT levels according to the etiology of febrile episodes

Among the 144 febrile episodes, 130 (90%) and 47 (33%) events revealed positivity for CRP and PCT, respectively. There was no difference in age or sex between patients with PCT positivity and those with PCT negativity (Table 1). PCT positivity was much more frequent in febrile episodes of HSCT patients (44%) than in those of non-HSCT patients (25%) (p=0.020). PCT positivity was associated with higher serum levels of CRP (p<0.0001). Although some studies have demonstrated that PCT levels are not markedly elevated in neutropenic patients (16, 17), in the present study ANC levels did not differ between patients showing PCT positivity and those with PCT negativity (Table 1).

We further evaluated the rates of CRP and PCT positivity for each etiology of fever. The incidence of CRP positivity was quite high both in infectious and non-infectious episodes, although a slight difference was observed (Fig. 1A; 96% vs 82%; p=0.005). On the other hand, the rates of PCT positivity were significantly higher in infectious episodes than in non-infectious episodes (41% vs. 21%, p=0.009, Fig. 1B), and especially high in patients with documented infections (61%). Of note, the rates of PCT positivity were quite low in FN, which were in contrast to the high rates of CRP positivity (23% and 94%, respectively). Among patients with documented infection, there was no difference between the level of CRP or semi-quantitative PCT and causative bacteria: median level of CRP and the number of patients categorized into 2+ or 3+ were similar in 12 gram-negative rod infections (CRP; 6.81 mg/dL, and PCT; 6/11) and in 11 gram-positive cocci infections (CRP; 6.37 mg/dL, and PCT; 4/11). Both of two candidemia patients revealed higher levels of CRP (14.88 and 28.23 mg/dL, respectively)

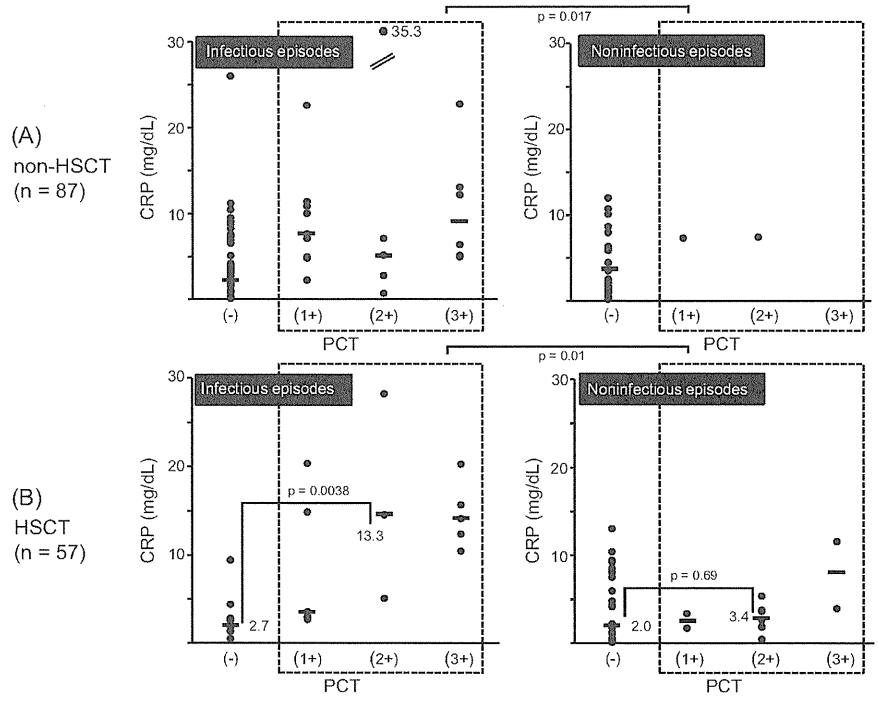

Figure 2. Association between the levels of CRP and semiquantitative PCT in (A) non-HSCT and (B) HSCT patient.

with positive results of PCT, while a case with adenovirus (ADV) infection revealed lower CRP value (4.81 mg/dL) with weak PCT positivity.

Results of PCT and CRP tests in HSCT and non-HSCT patients

Noninfectious febrile complications occur more frequently after HSCT than after conventional chemotherapy. Thus, we separately evaluated the diagnostic values of the tests for PCT and CRP in non-HSCT patients (n=57) and HSCT patients (n=87). Median ANC was comparable between the two groups (0.25×10^9/L vs. 0.17×10^9/L). No difference in the rate of CRP positivity (93% in non-HSCT vs. 86% in HSCT, p=0.157) or in the median CRP value between the groups (3.6 vs. 4.2 mg/dL, p=0.695) was observed. On the other hand, PCT positivity was more frequently observed in HSCT recipients than in non-HSCT patients (as shown in Table 1).

In non-HSCT patients, PCT positivity was observed in 20 of 60 (33%) infectious episodes and in 2 of 27 (7%) noninfectious episodes (p=0.01), demonstrating its good specificity for infections (Fig. 2A). The median CRP value in infectious episodes was marginally higher than its value in noninfectious episodes (4.9 vs. 2.5 mg/dL, p=0.067).

Among the 57 febrile events occurring after HSCT, 14 of 22 (64%) infectious episodes and 11 of 35 (31%) noninfectious episodes showed positive results for PCT (p=0.017) (Fig. 2B). It was found that nine of 11 noninfectious episodes with PCT positivity were due to allogeneic immune reactions (ES: n=2, GVHD: n=7). The association between GVHD severity and PCT levels, as demonstrated in a previous study (9), was not observed. CRP values were significantly higher in infectious episodes than in noninfectious episodes after HSCT (7.2 vs. 2.7 mg/dL, p=0.0035). Moreover, CRP values were substantially higher in patients showing PCT positivity than in those showing PCT negativity in infectious episodes (13.3 vs. 2.7 mg/dL, p=0.0038), but not in noninfectious episodes (3.4 vs. 2.0 mg/dL, p=0.69), after HSCT (Fig. 2B).

Diagnostic value of PCT and CRP for infection

PCT positivity of ≧1+ provided the best diagnostic value for infections compared to that of ≧2+ or 3+ in both HSCT and non-HSCT patients by the Youden index and AUC (data not shown).

In non-HSCT patients, sensitivity, specificity, PPV, and NPV of PCT positivity were 33%, 93%, 91%, and 39%, respectively (Table 2). The best cutoff value of serum CRP calculated from the ROC curve was 2.5 mg/dL (data not shown), and its sensitivity, specificity, PPV, and NPV were 77%, 48%, 77%, and 48%, respectively (Table 2). Univariate logistic analysis revealed that both PCT (p=0.019) and CRP (p=0.023) positivities were associated with infectious episodes, whereas multivariate analysis demonstrated that PCT positivity rather than CRP positivity (>2.5 mg/dL) could be a more reliable marker in a non-HSCT setting [Odds ratio (OR): 4.9 vs. 2.3; p value: 0.046 vs. 0.103, respectively] (Table 3).

Among HSCT patients, PCT positivity showed 64% sensitivity and 69% specificity (Table 2). In line with several

Table 2. Diagnostic Value of PCT and CRP

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Non-HSCT patients (n = 87)				
Positive results of PCT	33.3	92.6	90.9	38.5
Serum level of CRP > 2.5 mg/dL	76.7	48.1	76.7	48.1
HSCT patients (n = 57)				
Positive results of PCT	63.6	68.6	56.0	75.0
Serum level of CRP > 9.5 mg/dL	50.0	88.6	73.3	73.8

PPV indicates positive predictive value; NPV, negative predictive value;
PCT, procalcitonin; CRP, C-reactive protein

Table 3. Results of a Logistic Regression Analysis

			Non-HSCT patients (n = 87)		HSCT patients (n = 57)	
			Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
【Univariate analysis】	Sex	Male vs. Female	1.077 (0.434 - 2.672)	0.873	0.75 (0.257 - 2.188)	0.599
	Age	per 1years	0.981 (0.95 - 1.014)	0.257	1.003 (0.967 - 1.04)	0.866
	Underlying diseases	Myeloid (ref)	1		1	
		Lymphoid	1.667 (0.62 - 4.477)	0.311	0.787 (0.27 - 2.293)	0.661
		Others	3.333 (0.337 - 32.95)	0.303	-	-
	Neutrophil counts	per 1 x 10^9/L	0.925 (0.83 - 1.03)	0.157	0.926 (0.744 - 1.152)	0.49
	PCT	pos vs. neg	6.25 (1.344 - 29.07)	*0.019*	3.818 (1.241 - 11.75)	*0.02*
	CRP*	> vs. < cut-off	3.051 (1.164 - 7.994)	*0.023*	7.750 (2.039 - 29.46)	*0.003*
【Multivariate analysis】	PCT	pos vs. neg	4.943 (1.03 - 23.72)	***0.046***	2.86 (0.848 - 9.649)	0.09
	CRP*	> vs. < cut-off	2.297 (0.846 - 6.24)	0.103	6.268 (1.582 - 24.83)	***0.009***

PCT; procalcitonin, CRP; C-reactive protein
* Cut-off values were used as 2.5 and 9.5 mg/dL in non-HSCT and HSCT patients, respectively

previous studies that suggested the usefulness of considerably high cutoff levels of CRP (>10-15 mg/dL) to discriminate infectious from noninfectious complications (9, 18), the ROC curve defined the best cutoff value of CRP as >9.5 mg/dL in this study; its sensitivity, specificity, PPV, and NPV were 50%, 89%, 73%, and 74%, respectively (Table 2). Multivariate analysis revealed that CRP positivity with higher cutoff levels (>9.5 mg/dL) rather than PCT positivity provided superior diagnostic value for infectious complications in HSCT recipients (OR and p values were 6.3 vs. 2.9 and 0.009 vs. 0.09, respectively), although both markers showed correlation with infectious episodes in univariate analysis (Table 3).

Discussion

The present study showed that PCT is a considerably useful marker of infections in patients with hematological disorders and it helps discriminate documented infections from other febrile complications. Although almost all previous studies used a quantitative PCT measurement, we employed a semiquantitative assay that required only 30 minutes to accomplish, facilitating prompt therapeutic decision making. It takes from at least a day to a week for getting the results of other microbiological markers (e.g. cultured samples, galactomannan antigen, and β-D glucan), thus the rapid availability of the results of semiquantitative PCT as well as CRP could provide considerable advantage for both patients and clinicians.

Some previous studies (16, 18-21) have shown that PCT levels were higher in patients with gram-negative bacteremia than in those with gram-positive bacteremia, while the present study and another previous study (9) failed to show such an association. Two candidemia episodes showed positive results for PCT with high CRP levels in our study, as previously reported (9). It has been widely accepted that PCT was not elevated during viral infection (6, 22), probably in part due to the inhibitory effect of interferon-γ (IFN-γ) induced by viral infection on PCT gene transcription (23). However, PCT was weakly positive in a HSCT patient with ADV-associated hemorrhagic cystitis. It is possible that immunosuppressants used for GVHD prophylaxis inhibit IFN-γ production from T cells in response to infections or that there may be coexisting latent bacterial infections in these highly immunosuppressive patients. Thus, PCT positivity alone may not be helpful to estimate the causative pathogens particularly in HSCT patients.

Few episodes (7%) revealed false-positive results of PCT test in the non-HSCT setting, thus the diagnostic value of PCT was higher, with adequate specificity and PPV (93% and 91%, respectively), than that of CRP, as previously shown (6, 17, 18, 24). However, the sensitivity and NPV of PCT positivity were relatively low. In particular, the sensitivity of PCT was low in FN; PCT was positive in only 4 of 33 FN episodes. This may be because PCT is produced by leukocytes (25), although the sensitivity of the PCT assay remains controversial in FN patients (26-28). The timing of blood sampling may matter. A previous report demonstrated that the elevation of PCT was delayed compared to the immediate elevation of CRP (9), thereby suggesting that CRP

could have better diagnostic value than PCT in FN. Moreover, a recent investigation (29) has reported the different kinetics of PCT based on the causes of fever; the peak occurred on day 2 after the onset of fever in FN patients, while beyond 3 days in patients with invasive fungal diseases. We could not detect a specific regularity in PCT and CRP elevation during clinical course, although there was a limitation of retrospective analysis. PCT test has never turned to be positive in spite of their repeated higher levels of CRP in some cases, while others revealed PCT positivity following the gradual rise of CRP level.

In HSCT recipients, the diagnostic value of PCT to distinguish infections from other transplant-related complications remains controversial (9-12). The current study revealed that 30% of patients with GVHD or ES exhibited PCT positivity. Several studies suggested an association between PCT positivity and mucositis (21, 28). During GVHD, gastrointestinal damage permits the translocation of lipopolysaccharide that stimulates mononuclear cells to produce large amounts of inflammatory cytokines such as TNF-α (30). These inflammatory mediators stimulate leukocytes to produce PCT (25). We also observed PCT positivity during allergic reactions after anti-thymocyte globulin administration, as shown previously (31, 32). Thus, the specificity of PCT positivity to detect infections is not satisfactory in HSCT patients. We found that a high cutoff CRP level (>9.5 mg/dL) was statistically more diagnostic than PCT positivity, as has been shown previously (12). When high cutoff values of CRP were combined with PCT positivity, the specificity and PPV were further improved (98% and 94%, respectively) (data not shown), as previously reported (9).

There are several limitations for interpreting the results of our study. Selection bias is always a problem in retrospective studies. High false-positivity in HSCT patients may be due to masked infections in highly immunosuppressed patients, especially those with GVHD. In addition, this study could not address the influence of corticosteroid (33) or prophylactic antibiotic administration on the results of infectious biomarkers.

In conclusion, a semi-quantitative PCT test and CRP levels are useful biomarkers due to their higher diagnostic value and rapidity in patients with hematological disorders. The best cutoff CRP levels may differ between HSCT and non-HSCT patients. PCT is highly specific and statistically superior to CRP in non-HSCT cohorts. In HSCT cohorts, a combination of PCT and a high cutoff CRP level may be required, probably due to the higher PCT false-positivity in GVHD. Although these results should be confirmed in a prospective study including larger numbers of patients, we may have to interpret the results of these infectious markers with a different order of priority according to the treatment procedures.

The authors state that they have no Conflict of Interest (COI).

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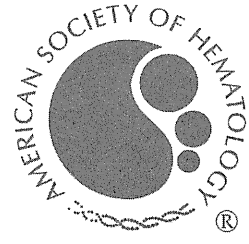

Regenerating islet-derived 3-alpha is a biomarker of gastrointestinal graft-versus-host disease

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There are no plasma biomarkers specific for GVHD of the gastrointestinal (GI) tract, the GVHD target organ most associated with nonrelapse mortality (NRM) following hematopoietic cell transplantation (HCT). Using an unbiased, large-scale, quantitative proteomic discovery approach to identify candidate biomarkers that were increased in plasma from HCT patients with GI GVHD, 74 proteins were increased at least 2-fold; 5 were of GI origin. We validated the lead candidate, REG3α, by ELISA in samples from 1014 HCT patients from 3 transplantation centers. Plasma REG3α concentrations were 3-fold higher in patients at GI GVHD onset than in all other patients and correlated most closely with lower GI GVHD. REG3α concentrations at GVHD onset predicted response to therapy at 4 weeks, 1-year NRM, and 1-year survival ($P \leq .001$). In a multivariate analysis, advanced clinical stage, severe histologic damage, and high REG3α concentrations at GVHD diagnosis independently predicted 1-year NRM, which progressively increased with higher numbers of onset risk factors present: 25% for patients with 0 risk factors to 86% with 3 risk factors present ($P < .001$). REG3α is a plasma biomarker of GI GVHD that can be combined with clinical stage and histologic grade to improve risk stratification of patients. (*Blood*. 2011;118(25): 6702-6708)

Introduction

Acute GVHD, a leading cause of nonrelapse mortality (NRM) after allogeneic hematopoietic cell transplantation (HCT), is measured by dysfunction in 3 organ systems: the skin, liver, and gastrointestinal (GI) tract.[1-4] Acute GVHD of the GI tract affects up to 60% of patients receiving allogeneic HCT,[5,6] causing nausea, vomiting, anorexia, secretory diarrhea, and, in more severe cases, abdominal pain and/or hemorrhage.[7] Acute GVHD typically occurs between 2 and 8 weeks after transplantation, but may occur later,[4] and is often clinically indistinguishable from other causes of GI dysfunction such as conditioning regimen toxicity, infection, or medication. Endoscopic biopsy is often used to confirm the diagnosis,[1,8] but histologic severity on biopsy has not consistently correlated with clinical outcome.[3,8-10] Clinical stage II or greater (> 1 L of diarrhea/d) is associated with reduced survival,[5,6] but daily stool volume can vary considerably. Lower GI GVHD responds poorly to treatment compared with other target organs,[6] and treatment with high-dose systemic steroid therapy carries significant risks, especially infectious complications in profoundly immunosuppressed patients.[11,12] A noninvasive, reliable blood biomarker specific for GVHD of the GI tract would thus significantly aid in the management of patients with this disorder.

Here, we report the discovery and validation of a plasma biomarker of acute GI GVHD: regenerating islet-derived 3-alpha (REG3α), a C-type lectin secreted by Paneth cells.[13,14]

Methods

Proteomic analysis

Methods for sample preparation, protein fractionation, mass spectrometry (MS) analysis, protein identification, and quantitative analysis of protein concentrations during the intact protein analysis system (IPAS) have been previously reported.[15-17]

Patients and samples

Heparinized blood samples were collected weekly for 4 weeks after allogeneic HCT, then monthly for 2 months, and also at the time of key clinical events, including the development of symptoms consistent with GVHD (eg, the onset of diarrhea). Plasma samples were collected prospectively under protocols approved by the University of Michigan Institutional Review Board and stored at the University of Michigan. GVHD assessments, sample processing, and storage were performed as previously described.[7,17] In Regensburg, Germany, and Kyushu, Japan, serum samples were collected weekly and at the onset of GVHD symptoms, prepared, frozen, and stored per institutional guidelines. Samples were shipped and received frozen on dry ice and no sample was thawed more than twice before analysis. REG3α concentrations were stable in samples frozen for at least 5 years. REG3α concentrations of 12 paired healthy donors plasma and serum were similar (mean ± SEM: 20 ± 3 vs 24 ± 3 ng/mL, respectively).

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Table 1. Patient characteristics of the University of Michigan validation set

Total, N = 871	GI GVHD*†, N = 167	No GVHD, N = 362	Non-GVHD enteritis‡, N = 52	Skin GVHD, N = 290	*P*
Median age, y (range)	50 (0-67)	46 (0-68)	48 (3-66)	49 (0-70)	.003
Disease, %					.002
Malignant	99 (n = 165)	92 (n = 334)	96 (n = 50)	97 (n = 282)	
Other	1 (n = 2)	8 (n = 28)	4 (n = 2)	3 (n = 8)	
Disease status at transplantation, %§					.63
Other/low/intermediate risk	64 (n = 105)	69 (n = 232)	68 (n = 34)	68 (n = 192)	
High risk	36 (n = 60)	31 (n = 102)	32 (n = 16)	32 (n = 90)	
Donor type, %					< .001
Related donor	45 (n = 75)	64 (n = 233)	54 (n = 28)	40 (n = 115)	
Unrelated donor	55 (n = 92)	36 (n = 129)	46 (n = 24)	60 (n = 175)	
Donor match, %					< .001
Matched donor	70 (n = 117)	90 (n = 325)	92 (n = 48)	73 (n = 212)	
Mismatched donor	30 (n = 50)	10 (n = 37)	8 (n = 4)	27 (n = 78)	
Conditioning regimen intensity, %					.06
High intensity	57 (n = 95)	67 (n = 243)	63 (n = 33)	57 (n = 165)	
Moderate intensity	43 (n = 72)	33 (n = 119)	37 (n = 19)	43 (n = 125)	
Grade of GVHD at onset, %					
0	0 (n = 0)	100 (n = 362)	100 (n = 52)	0 (n = 0)	
I	0 (n = 0)	0 (n = 0)	0 (n = 0)	69 (n = 201)	
Skin stage 1	0 (n = 0)	0 (n = 0)	0 (n = 0)	41 (n = 118)	
Skin stage 2	0 (n = 0)	0 (n = 0)	0 (n = 0)	29 (n = 83)	
II	57 (n = 96)	0 (n = 0)	0 (n = 0)	30 (n = 88)	
Isolated skin stage 3	0 (n = 0)	0 (n = 0)	0 (n = 0)	30 (n = 88)	
Isolated upper GI stage 1†	17 (n = 29)	0 (n = 0)	0 (n = 0)	0 (n = 0)	
Lower GI stage 1†	40 (n = 67)	0 (n = 0)	0 (n = 0)	0 (n = 0)	
III-IV	43 (n = 71)	0 (n = 0)	0 (n = 0)	1 (n = 1)	
Isolated skin stage 4	0 (n = 0)	0 (n = 0)	0 (n = 0)	1 (n = 1)	
GI stage 2†	13 (n = 22)	0 (n = 0)	0 (n = 0)	0 (n = 0)	
GI stage 3†	16 (n = 27)	0 (n = 0)	0 (n = 0)	0 (n = 0)	
GI stage 4†	13 (n = 22)	0 (n = 0)	0 (n = 0)	0 (n = 0)	
Median d after HCT (range)	33 (11-216)	31 (7-185)	24 (7-93)	28 (5-175)	< .001

GI indicates gastrointestinal; HCT, hematopoietic cell transplantation; and CIBMTR, Center for International Blood and Marrow Transplant Research.
*Including 29 patients with isolated upper GI GVHD and 138 with lower ± upper GI GVHD.
†With or without other GVHD target organ involvement.
‡Including 13 patients with isolated upper GI non-GVHD enteritis and 39 patients with lower ± upper GI non-GVHD enteritis.
§High risk of disease status at HCT is according to CIBMTR guidelines.

All patients received pharmacologic GVHD prophylaxis with at least 2 agents, including a calcineurin inhibitor. No donor grafts were depleted of T cells. All patients with available samples were analyzed, including patients who developed other complications of HCT, such as sinusoidal obstruction syndrome (SOS), idiopathic pneumonia syndrome (IPS), and sepsis/bacteremia. Patients were excluded from analysis only if a plasma sample at the time of GVHD onset was not available, or if methylprednisolone > 1 mg/kg (or equivalent) had been administered for > 48 hours at the time of sample acquisition. One sample was analyzed per patient; patients who developed GVHD had samples selected at the time of initial GVHD diagnosis.

The discovery set consisted of plasma samples from 10 HCT patients at the onset of biopsy-proven GI GVHD (clinical stage 1-3) and 10 HCT patients who never developed GVHD and who were matched for key transplantation characteristics (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Patient samples in the discovery set were not included in the validation set.

The University of Michigan validation set consisted of 4 groups: patients with newly diagnosed GVHD involving the GI tract (with or without other organ involvement; GI GVHD); patients at similar time points who never developed GVHD symptoms (no GVHD); patients with GI distress that was inconsistent with GVHD either by clinical or histologic criteria (non-GVHD enteritis); and patients who presented with isolated skin GVHD (skin GVHD). Patient numbers and characteristics are shown in Table 1. Enteritis was determined to be inconsistent with GVHD on clinical grounds by documentation of infected stool and by resolution of symptoms without steroid treatment. The etiologies of non-GVHD enteritis are listed in Table 2.

Patients from the Regensburg/Kyushu validation set were divided into the same 4 groups; patient characteristics are detailed in supplemental Table 2, with causes of non-GVHD enteritis listed in supplemental Table 3.

Histopathology

GI biopsies were obtained and prepared per institutional guidelines. GVHD was histologically confirmed by duodenal/colonic biopsy in 183 of 197 GI GVHD patients and by skin biopsy in an additional 5 patients with both rash

Table 2. Causes of non-GVHD enteritis in the University of Michigan validation set

Causes of non-GVHD enteritis	% (n)
Non-GVHD lower GI enteritis ± upper GI symptoms: N = 39	
Clostridium difficile infection	54 (21)
Diarrhea with negative biopsy	15 (6)
Nausea/vomiting and diarrhea with negative biopsies	28 (11)
Ulcerative esophagitis and diarrhea (negative biopsies)	3 (1)
Non-GVHD upper GI enteritis without diarrhea (all biopsy negative): N = 13	
Nausea/vomiting	54 (7)
Anorexia	15 (2)
Chemical gastropathy	23 (3)
Helicobacter pylori gastritis	8 (1)

GI indicates gastrointestinal.

and GI symptoms.[9] Skin GVHD was confirmed by biopsy in 272 of 341 patients with rashes and by biopsy of another target organ later affected by GVHD in an additional 8 patients. One hundred sixty-two of 197 patients with GI GVHD had diarrhea. One hundred forty of those 162 patients had biopsies (duodenal = 87, colonic = 53) available for formal grading as described by Lerner.[18] If both duodenal and colonic biopsies were available, colonic biopsies were graded only if duodenal biopsies were negative. We did not impute values for unavailable biopsies.

ELISAs

REG3α ELISA kits were purchased from MBL International (Ab-Match Assembly Human PAP1 kit and Ab-Match Universal kit), and measurements were performed according to the manufacturer's protocol. Samples (diluted 1:10) and standards were run in duplicate, absorbance was measured with a SpectraMax M2 (Molecular Devices), and results were calculated with SoftMax Pro Version 5.4 (Molecular Devices). Elafin, IL2Rα, HGF, TNFR1, and IL-8 ELISAs were performed in duplicate as previously reported.[17,19] Measurements of samples from 66 patients (6.5% of the total population) were repeated in a second ELISA at random intervals and were comparable; correlation coefficient r = 0.82, $P < .0001$. Details of the assay parameters are provided in supplemental Table 4.

Statistical analysis

The statistical methods used for the IPAS are as previously described.[15-17] REG3α and albumin concentrations from individual samples in the discovery and validation sets were compared using 2-sample *t* tests applied to log-transformed concentrations. Differences in characteristics between patient groups were assessed with a Kruskal-Wallis test for continuous values and χ^2 tests of association for categorical values. Receiver operating characteristic (ROC) area under the curves (AUC) were estimated nonparametrically. NRM and relapse mortality were modeled with cumulative incidence regression methods as described by Fine and Gray.[20] One-year overall survival (OS) was modeled with Cox regression methods and probability of response was modeled with logistic regression.

Results

Discovery study

We used a proteomics approach to identify candidate biomarkers in a discovery set of pooled plasma samples taken at similar times after HCT from 10 patients with biopsy-proven GI GVHD and 10 patients without GVHD as previously described (supplemental Table 1).[15-17] We identified and quantified 562 proteins of which 74 were increased at least 2-fold in patients with GVHD (supplemental Table 5). Five proteins (carboxypeptidase N catalytic chain precursor, pancreatic secretory trypsin inhibitor precursor, palladin, lithostathine 1-α precursor, and regenerating islet-derived 3-alpha) were preferentially expressed in the GI tract based on the relevant literature[21-25] and the Human Protein Atlas (http://www.proteinatlas.org/). Commercially available Abs suitable for quantification of plasma concentrations by ELISA were available for only 1 of these 5 proteins, regenerating islet-derived 3-α (REG3α; supplemental Table 5). The MS characteristics of the identified REG3α peptides are shown in supplemental Figure 1 and supplemental Table 6. The plasma concentrations of REG3α in the individual plasma samples in the discovery set were 4 times higher in the patients with GI GVHD than in asymptomatic controls (supplemental Figure 2, $P = .01$).

Validation study

We next evaluated REG3α plasma concentration as a biomarker of GI GVHD in samples from a validation set of 871 allogeneic HCT recipients from the University of Michigan (Table 1). Older transplant recipients, an underlying diagnosis of malignant disease, graft sources from unrelated and HLA-mismatched donors were overrepresented in the groups with GVHD. The median day of sample acquisition for patients with non-GVHD enteritis was closer to the day of transplantation than for all other groups.

Plasma REG3α concentrations were 3 times higher in patients at the onset of GI GVHD than in all other patients, including those with non-GVHD enteritis (Figure 1A). There was no specific cause of non-GVHD diarrhea associated with higher REG3α concentrations. Serum REG3α concentrations were also higher in GI GVHD in an independent validation set of 143 HCT patients from Regensburg, Germany, and Kyushu, Japan, although the absolute values were lower (Figure 1B). This difference may be because of a center effect that depends on several factors, including variations in transplantation conditioning regimens and supportive care; patients receiving high-intensity conditioning regimens had REG3α concentrations that were twice as high as those receiving moderate intensity conditioning, but this difference did not reach statistical significance (Figure 1C). In addition, all patients in Regensburg and Kyushu received oral antibiotics as GVHD prophylaxis, whereas Michigan patients did not and thus increased GI flora might account for greater REG3α secretion.[26] Neither total body irradiation (TBI)–based conditioning nor GVHD prophylaxis regimen significantly impacted REG3α concentrations (data not shown).

We next analyzed REG3α concentrations according to diagnosis and type of GI symptom. In patients with diarrhea caused by GVHD, REG3α concentrations at the onset of GVHD were 5 times higher than in patients with diarrhea from other causes (Figure 1D). In patients without diarrhea, REG3α concentrations were 25% higher when attributable to GVHD compared with other causes, a difference that was not statistically significant.

We measured concentrations of 4 previously reported diagnostic markers of systemic acute GVHD (IL2Rα, TNFR1, IL-8, and HGF),[19] and of elafin, a biomarker for GVHD of the skin,[17] in all patients with diarrhea (Figure 1C, N = 204). ROC curves for these biomarkers distinguished GVHD from non-GVHD with an AUC of 0.80 for REG3α alone and an AUC of 0.81 for a composite panel of all 6 biomarkers (Figure 2). In this analysis, 52% of patients with lower GI GVHD also had skin involvement at onset, and thus the AUC for elafin, which is specific for GVHD of the skin,[17] was greater than expected (supplemental Table 7). ROC curves of REG3α concentrations in patients with diarrhea had similar AUCs in both validation sets (supplemental Figure 3). REG3α was therefore the best single diagnostic biomarker at the onset of symptoms of lower GI GVHD, and additional biomarkers provided no further increased sensitivity or specificity. Using REG3α at the median concentration provided a positive predictive value (PPV) of 95% and a negative predictive value (NPV) of 32% for GVHD as the etiology of diarrhea. Additional predictive values at other REG3α concentrations are provided in supplemental Table 8.

When we categorized patients by the volume of diarrhea, REG3α concentrations at the onset of symptoms continued to distinguish between GVHD and non-GVHD etiologies (Figure 3A, $P < .001$) but did not correlate with the clinical stage of GVHD. Twenty-three of 26 patients with clinical stage IV GI GVHD at onset received full-intensity conditioning, and these patients showed a trend toward higher REG3α concentrations than those with stage 1–3 GI GVHD ($P = .07$; data not shown). Comparing patients who had < 1 L of stool per day because of GVHD versus other causes, the AUC for REG3α was 0.81 (supplemental Figure 4). Plasma REG3α concentrations at the onset of GVHD were significantly

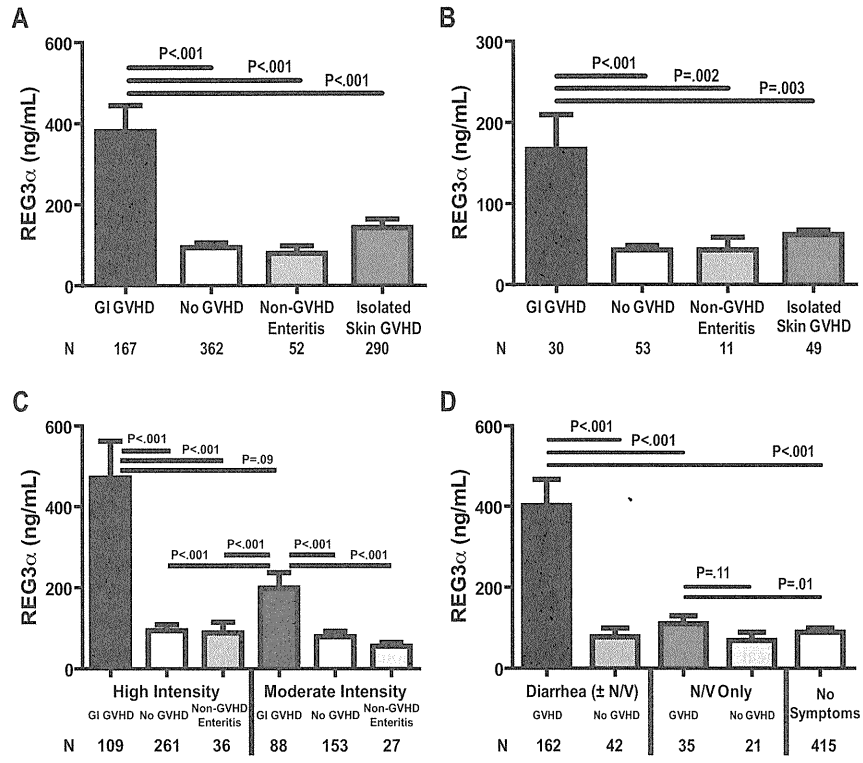

Figure 1. REG3α concentrations in plasma samples from HCT patients of 2 independent validation sets. (A) University of Michigan patients (n = 871). (B) Regensburg, Germany, and Kyushu, Japan (n = 143). (C) Plasma REG3α concentrations in patients classified by GI symptoms and histologic diagnosis and categorized by conditioning regimen intensity. High-intensity regimens included: cyclophosphamide ± cytarabine, thiotepa, fludarabine and/or TBI; cyclophosphamide/VP-16/BCNU; busulfan + cytarabine, clofarabine, melphalan, cyclophosphamide/anasacrin, or cytarabine/cyclophosphamide; BCNU/VP-16/cytarabine/melphalan; TBI ± VP-16; melphalan. Moderate-intensity regimens included: fludarabine + busulfan or treosulfan ± TBI, melphalan, zevalin, or anasacrin/cytarabine; fludarabine ± TBI, melphalan, or cyclophosphamide; fludarabine/BCNU/melphalan; TBI. (D) Patients classified by symptoms and etiology (n = 675).

higher in patients whose GI biopsies showed evidence of severe GVHD with mucosal denudation (histologic grade 4) compared with less severe GVHD (Figure 3B; $P = .03$). Hypoalbuminemia is associated with the protein-losing enteropathy in GI GVHD,[27] and we analyzed the serum albumin level as a potential marker for loss of intravascular proteins into the intestinal lumen. Albumin levels at the onset of GI GVHD also correlated with both the clinical GI GVHD severity (supplemental Figure 5A) and histopathologic severity (supplemental Figure 5B).

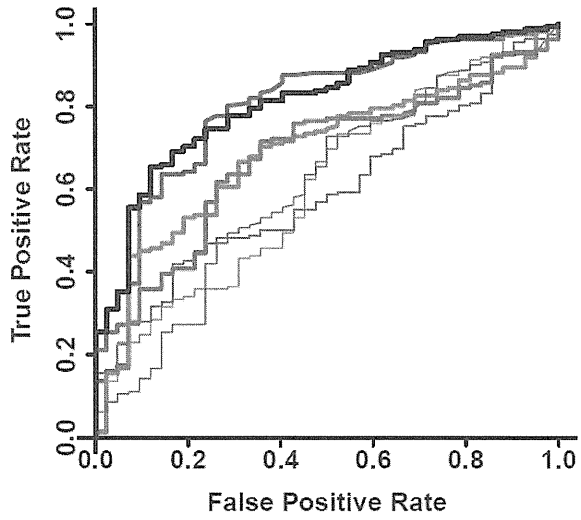

Figure 2. ROC curves for patients with post-HCT diarrhea. ROC curves comparing REG3α concentrations for patients with diarrhea caused by GVHD (n = 162) and not caused by GVHD (N = 42). REGα alone (thick blue): AUC = 0.80; IL2Rα (thick brown): AUC = 0.69; Elafin (thick red): AUC = 0.68; IL-8 (thin blue): AUC = 0.61; HGF (thin brown): AUC = 0.61; TNFR1 (thin red): AUC = 0.60; composite of all 6 biomarkers (solid black): AUC = 0.81.

Prognostic value of REG3α concentrations in patients with lower GI GVHD

The clinical use of any biomarker is greatly enhanced when it provides prognostic information regarding the future status of a disease and/or patient, for example, the likelihood of response to treatment. We therefore evaluated the prognostic significance of REG3α plasma levels in 162 patients taken at the time of diagnosis of lower GI GVHD. REG3α concentrations were 3-fold higher at the time of GVHD diagnosis in patients who had no response to therapy at 4 weeks[28,29] than in patients who experienced a complete or partial response (Figure 4A; $P < .001$)[28,29]; patients responding to therapy still exhibited REG3α concentrations more than twice that of non-GVHD controls. REG3α concentrations at diagnosis also correlated with eventual maximal clinical stage of GI GVHD (supplemental Figure 6); patients presenting with isolated skin GVHD who later developed GI GVHD had concentrations comparable with those with skin GVHD who never developed GI GVHD ($P = .2$; data not shown). Because maximal GVHD grade correlates with NRM,[11] we hypothesized that the REG3α concentration at GVHD diagnosis would also correlate with NRM. We therefore divided the 162 patients into 2 equal groups based on the median REG3α concentration: high (> 151ng/mL, n = 81) and low (≤ 151 ng/mL, N = 81). NRM was twice as high in patients with high REG3α concentrations, and this difference remained significant after adjusting for known risk factors of donor type, degree of HLA match, conditioning intensity, age, and baseline disease severity (59% [95% confidence interval [CI], 48%-69%] vs 34%[95% CI, 24%-46%], $P < .001$, Figure 4B). The incidence of relapse mortality was comparable for both groups (14% [95% CI, 8-24] vs 17% [95% CI, 8-24], $P = .5$; Figure 4C), and thus patients with high REG3α concentrations at the time of GVHD diagnosis experienced significantly inferior 1-year OS (27% [95% CI, 19%-39%] vs 48% [95% CI, 38%-61%], $P = .001$; Figure 4D).

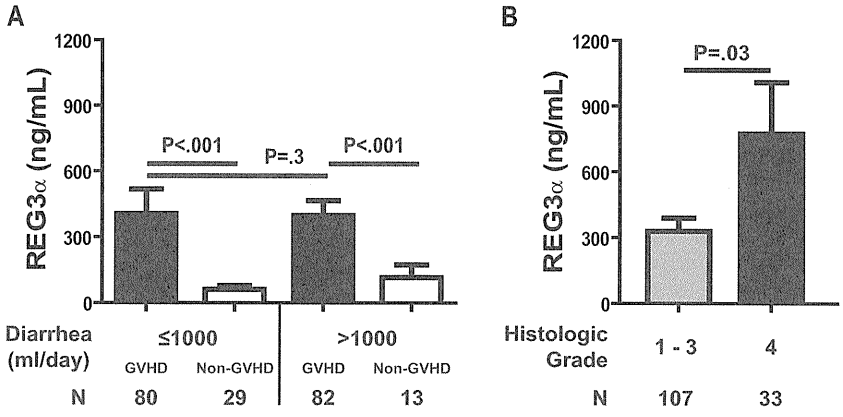

Figure 3. REG3α expression according to severity of GVHD at diagnosis. Patients were classified by volume of diarrhea (A) and histologic grade (B).

Causes of 1-year mortality for these patients are listed in supplemental Table 9.

Of the 162 patients with diarrhea at the onset of GVHD, we possessed all 4 data points of clinical stage, histologic grade, REG3α concentration, and serum albumin level in 140 patients. As shown in Table 3, the plasma concentration of REG3α, the clinical severity of GVHD, the histologic severity, and serum albumin level at GVHD diagnosis independently predicted lack of response to GVHD therapy 4 weeks following treatment after adjustment for the aforementioned risk factors (odds ratios: 4.8, 3.9, 18.9, and 2.5, respectively). When lack of response to therapy and NRM were modeled simultaneously on all 4 parameters, all but albumin remained statistically significant. When only advanced clinical stage and severe histologic grade were considered, NRM was 71% (Figure 4E). The inclusion of high REG3α concentration further risk-stratified patients who had either advanced clinical

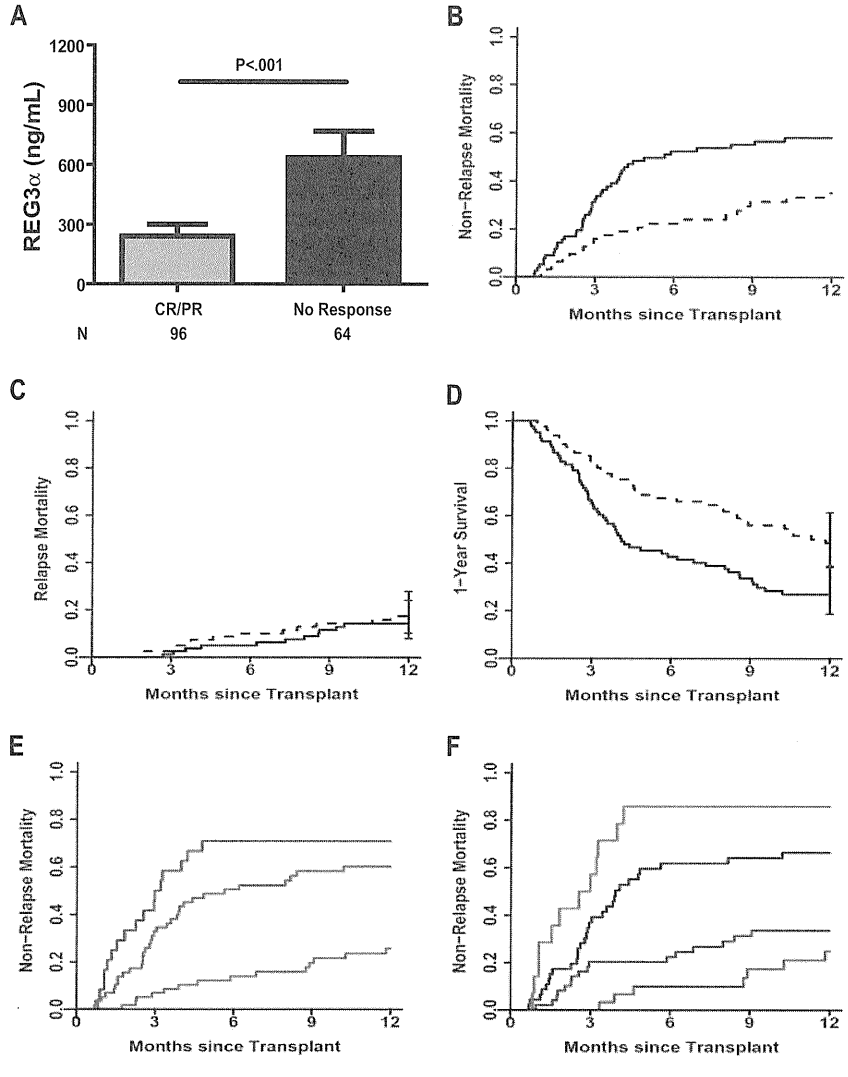

Figure 4. Prognostic value of REG3α concentrations at onset of GVHD. (A) Patients were classified by response to GVHD therapy after 4 weeks (N = 160). (B-D) Patients were classified by REG3α concentration: low (≤ 151 ng/mL, n = 81; thin line) and high (> 151 ng/mL, n = 81; thick line). (B) NRM (34% vs 59%, $P < .001$) (C) Relapse mortality (17% vs 14%, $P = .59$). (D) One-year survival (48% vs 27%, $P = .001$). All P values are adjusted for donor source, HLA match, conditioning intensity, recipient age, and baseline disease severity according to the Center for International Blood and Marrow Transplant Research (CIBMTR) guidelines. (E) One-year NRM for patients classified by number of risk factors at GVHD onset, using clinical stage (high risk = stage 2-4) and histologic grade (high risk = grade 4); 0 (purple, NRM = 26%); 1 (red, NRM = 60%); 2 (blue, NRM = 71%); 0 vs 1, $P < .001$; 1 vs 2, $P = .006$. (F) One-year NRM for patients classified by number of risk factors at the time of GVHD diagnosis as in panel E and including REG3α concentration (high risk > 151 ng/mL); 0 (purple, NRM = 25%); 1 (red, NRM = 34%); 2 (purple, NRM = 66%); 3 (brown, NRM = 86%); 0 vs 1, $P = .2$; 1 vs 2, $P < .001$; 2 vs 3, $P < .001$.

Table 3. REG3α concentrations and characteristics at onset of GVHD diarrhea predict 4-week response to GVHD therapy and 1-year NRM

	Independent		Simultaneous	
	Ratio	P*	Ratio	P*
No response to treatment (at 4 wk)	Odds		Odds	
REG3α (high vs low)	4.8	< .001	5.7	.001
GVHD GI onset stage (2-4 vs 1)	3.9	.001	3.0	.027
Histologic grade (4 vs 1-3)	18.9	< .001	16.7	< .001
Albumin (low vs high)	2.5	.02	1.4	.5
1-y NRM	Hazard		Hazard	
REG3α (high vs low)	2.2	.003	2.4	.002
GVHD GI onset stage (2-4 vs 1)	3.0	< .001	3.1	< .001
Histologic grade (4 vs 1-3)	3.6	< .001	2.9	< .001
Albumin (low vs high)	2.3	.004	1.6	.2

NRM indicates nonrelapse mortality; and GI, gastrointestinal.
*Adjusted for age, donor type, HLA match, conditioning intensity, and disease status at transplantation.

stage or histologic severity (Figure 4F; 34% vs 66% for 1 or 2 risk factors, respectively, $P < .001$), and patients who had all 3 risk factors experienced significantly greater NRM than those with any 2 of the risk factors (86% vs 66%, $P < .001$). Details of patient risk factors are listed in supplemental Table 10; NRM by all other risk factor combinations are shown in supplemental Figure 7.

Discussion

The etiology of diarrhea following HCT presents a common diagnostic dilemma.[30,31] We identified REG3α as a candidate biomarker specific for lower GI GVHD through an unbiased, in-depth tandem MS-based discovery approach that can quantify proteins at low concentrations and that we previously used successfully to identify elafin as a plasma biomarker specific for GVHD of the skin.[17] Our discovery approach identified 74 proteins that were increased at least 2-fold in the plasma from patients with GI GVHD. Of note, the list did not include cytokeratin-18 (KRT18), which has been reported to be specific for both liver and intestinal GVHD.[32] This discrepancy may be explained by limitations in proteomics technology and the significantly later acquisition times of samples in the earlier report.

REG proteins act downstream of IL-22 to protect the epithelial barrier function of the intestinal mucosa[33,34] through the binding of bacterial peptidoglycans.[13] Intestinal stem cells (ISCs) are principal cellular targets of GVHD in the GI tract,[3,35] where intestinal flora are critical for amplification of GVHD damage.[36,37] A leading hypothesis is that ISCs are protected by antibacterial proteins such as REG3α secreted by neighboring Paneth cells into the crypt microenvironment.[38] If death of an ISC eventually manifests itself as denudation of the mucosa, the patchy nature of GVHD histologic damage may be explained as the lack of mucosal regeneration following the dropout of individual ISCs.[3,35] REG3α reduces the inflammation of human intestinal crypts in vitro,[14,39] and its administration protects ISCs and prevents GI epithelial damage in vivo,[34] raising interesting therapeutic possibilities for this molecule.

REG3α plasma concentrations correlate with disease activity in inflammatory bowel disease, and can distinguish infectious and autoimmune causes of diarrhea.[14] The correlation of mucosal denudation (histologic grade 4) with high REG3α concentrations suggests that microscopic breaches in the mucosal epithelial barrier caused by severe GVHD permit REG3α to traverse into the systemic circulation. The tight proximity of Paneth cells with ISCs concentrates their secretory contents in that vicinity, so that mucosal barrier disruption caused by stem cell dropout may preferentially allow Paneth cell secretions, including REG3α, to traverse into the bloodstream. We hypothesize that plasma levels of REG3α may therefore serve as a surrogate marker for the cumulative area of these breaches to GI mucosal barrier integrity, a parameter impossible to measure by individual tissue biopsies. Such an estimate of total damage to the mucosal barrier may also help explain the prognostic value of REG3α with respect to therapy responsiveness and NRM.

In this study, 3 high-risk parameters each independently correlated with lack of response to treatment and to higher NRM: elevated plasma REG3α concentration, higher clinical stage of GVHD at diagnosis and grade 4 histologic severity. All 3 of these values thus provided important prognostic information before the initiation of therapy rather than at the time of maximum grade of GVHD, which by definition includes responsiveness to therapy.[5,6,11] This study confirms earlier reports where higher clinical stage of GI GVHD[5,6] and more severe histology correlated with worse survival.[10] In our study the 1-year NRM was 33% (22 of 67 patients) in patients with clinical stage I lower GI GVHD when considering clinical severity alone. Seven of 8 patients (88%) who had the 2 other high risk factors present experienced 1-year NRM while 25% (15 of 59) of patients with 1 or no risk factors experienced 1-year NRM. In this regard it should be noted that REG3α levels did not obviate the need for biopsy. If the prognostic value of REG3α is confirmed in additional patients, we believe the integration of clinical stage, histologic grade and REG3α plasma concentrations into a single grading system will permit better risk stratification and rapid identification of those patients with severe GI damage in whom standard treatment is likely to be insufficient.

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Authorship

Contribution: J.L.M.F. planned the study, interpreted the data, and wrote the manuscript; A.C.H. designed and planned the experiments, performed research, performed data collection and quality assurance, analyzed data, and wrote the manuscript; J.K.G. and E. Huber performed pathology evaluations and wrote the manuscript; T.M.B. was the study statistician and wrote the manuscript; E. Holler, T.T., J.E.L., S.W.J.C., K. L., K.A., and P.R. contributed to patient accrual, clinical data collection and quality assurance, research discussion, and wrote the manuscript; M.V.L. performed experiments and wrote the manuscript; A.C., Q.Z., and S.H. performed the proteomics experiments, interpreted data, and wrote the manuscript; and S.P. conceived and planned the study design, performed experiments, interpreted data, and wrote the manuscript.

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Letter to the Editor

Expansion of NK cells from cord blood with antileukemic activity using GMP-compliant substances without feeder cells

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Neonatal cord blood (CB) cells have been demonstrated to contain a high percentage of natural killer (NK) cells, but the NK cells are immature, with a low level of cytolytic activity. However, expression levels of perforin and granzyme have been reported to be high in CB NK cells, and it has been suggested that CB NK cells are phenotypically and functionally mature. It has been suggested that CB is a source of stem cells that is as safe and effective as bone marrow or mobilized peripheral blood.[1,2] Also, there are a number of progenitor cell populations in CB that can be differentiated to NK cells. Therefore, CB is a useful source to expand NK cells for adoptive immunotherapy, particularly against malignant cells that express a low level of human leukocyte antigen class I molecules. Resting CB NK cells rapidly respond to cytokine stimulation by increasing cytolytic activity. Adoptive transfer of allogeneic NK cells is a potential immunotherapy to induce a graft-versus-leukemia (GVL) effect, without causing a graft-versus-host disease (GVHD).[3] In this study, we tried to expand NK cells from CB with antileukemic activity using good manufacturing practice (GMP)-compliant substances without feeder cells. We used tacrolimus (FK506) and low molecular weight heparin (dalteparin sodium) for expansion of NK cells. Kim *et al.*[4] showed impaired interleukin (IL)-2 signaling and a reduction in activating receptors in NK cells by tacrolimus. However, we have reported that tacrolimus enhances the cytolytic activity of inhibitory NK cell receptor (CD94/NKG2A)-expressing CD8T cells.[5] Also, Wang *et al.*[6] showed that a related compound (cyclosporine A) has essentially no effect on cytolytic activity of NK cells. There is conflicting data on the effect of calcineurin inhibitors on NK cell. Anyways, tacrolimus can inhibit T-cell proliferation. Heparin was reported to bind to several types of cytokines and to activate them, and also to have an important role in the expansion of hematopoietic progenitor cells.[7] Also, heparin sulfate and its related compounds were recognized by natural cytotoxicity receptors such as NKp30, NKp44 and NKp46, and soluble heparin enhanced the secretion of interferon-γ by NK cells.[8] Spanholtz *et al.*[9] reported efficient expansion of NK cells from CB $CD34^+$ cells, using low molecular weight heparin-based media containing various cytokines. Therefore, we tried to use tacrolimus to inhibit T-cell proliferation and dalteparin to support NK-cell proliferation during NK-cell expansion using IL-2, IL-15 and OKT3 *in vitro*.

Umbilical CB cells (Hokkaido Cord Blood Bank, Sapporo, Japan; 1×10^6 per ml) were cultured with IL-15 (10 ng/ml; PeproTech Inc., Rocky Hill, NJ, USA), IL-2 (5 ng/ml; R&D Systems, Minneapolis, MN, USA) and anti-CD3 monoclonal antibody (mAb) (OKT3, 10-1000 ng/ml, Janssen Pharmaceutical Company, Tokyo, Japan), with or without tacrolimus (0.02-0.1 ng/ml, Fujisawa, Osaka, Japan) and dalteparin sodium (Fragmin, 5-10 U/ml, Pfizer Japan, Tokyo, Japan) in culture medium stem cell growth medium (SCGM) (CeeGenix, Freiburg, Germany), which was produced under GMP, with 5% human AB serum in 24-well plates or T25 flasks without feeder cells. Cell cultures were split approximately one-second to one-fourth after 3-4 days of culture, and fresh medium, cytokines and reagents were added. After 3 weeks culture of umbilical CB cells (1×10^6 per ml) with IL-15, IL-2 and anti-CD3 mAb without feeder cells, $CD56^+CD3^-$ NK cells had increased by more than 1000-fold with about 50% purity. Furthermore, addition of dalteparin sodium and tacrolimus efficiently augmented NK cell expansion (1700-fold expansion with 72.8% purity). Also, NK cell proportion was the highest (72.8%) after expansion with both dalteparin sodium and tacrolimus compared with expansion with cytokines only, dalteparin sodium only and tacrolimus only (Table 1, means ± s.d.s, $n=5$). The proportion of $CD56^+CD3^-$ NK cells increased after more than 7 days of culture, and the proportion of CD56-expressing cells increased up to 90% after 3 weeks of culture (Figure 1a, bars indicate means ± s.d.s, $n=5$). Finally, we could obtain about 40×10^6 NK cells from 1×10^6 unmanipulated CB cells under GMP-conditioned medium with 5% human AB serum without feeder cells. Furthermore, this method has also enabled to expand NK cells from adult peripheral blood mononuclear cells (PBMCs; preliminary data not shown).

These expanded NK cells expressed stimulatory NK cell receptor NKG2D and intracellular cytotoxic molecule granzyme (Figure 1b, bars indicate means ± s.d.s, $n=5$). Also, the expanded $CD16^+CD56^+$ NK cells expressed high levels of inhibitory NK receptors, but significantly higher levels of stimulatory NK cell receptors including NKG2C, NKG2D, NKp30, NKp46 and especially, NKp44, than the levels of these receptors on $CD16^+CD56^+$ NK cells in resting CB before culture were noted (Figure 1c, $P<0.01$, bars indicate means ± s.d.s, $n=10$).

The cytolytic activities of expanded NK cells were tested against ^{51}Cr-labeled K562 human leukemic cell lines, patients' leukemic cells and allogeneic phytohemagglutin (PHA) blasts (5×10^3), using standard 4-h ^{51}Cr release assays. The expanded NK cells had

Table 1. Expansion of NK cells from CB samples

	Pre	*IL2+15*	*+Tacrolimus*	*+Fragmin*	*+T+F*
%	2.8 ± 0.8	48.3 ± 6.5	50.4 ± 3.7	65.0 ± 10.8[b]	72.8 ± 9.6[a,c,d]
Absolute number	0.028 ± 0.008	36.1 ± 10.4	34.7 ± 16.2	49.9 ± 17.7[e]	43.5 ± 14.3
Fold expansion	1	1422 ± 316	1360 ± 581	1989 ± 678[e]	1706 ± 389

Abbreviations: CB, cord blood; IL, interleukin; NK, natural killer. Values in the upper column indicate the percentage of $CD56^+CD3^-$ cells after 3-weeks expansion of CB cells with indicated factors (%). Significant differences were found in the values compared with the values for culture with only IL-2 and IL-15. Significant differences were also found in the values compared with the values for culture with 0.02 ng/ml of tacrolimus and 5 IU/ml of fragmin. Values in the middle and lower columns indicate the calculated absolute $CD56^+CD3^-$ cell number and fold expansion of NK cells after 3-weeks expansion of 1×10^6 CB cells. Significant difference was found only in the value for culture with IL-2, IL-15 and 0.02 ng/ml of tacrolimus, compared with the values for culture with IL-2, IL-15 and 5 IU/ml of fragmin (means ± s.d.s, $n=5$). [a]$P<0.01$, [b]$P<0.05$, [c]$P<0.01$, [d]$P<0.05$ and [e]$P<0.05$.

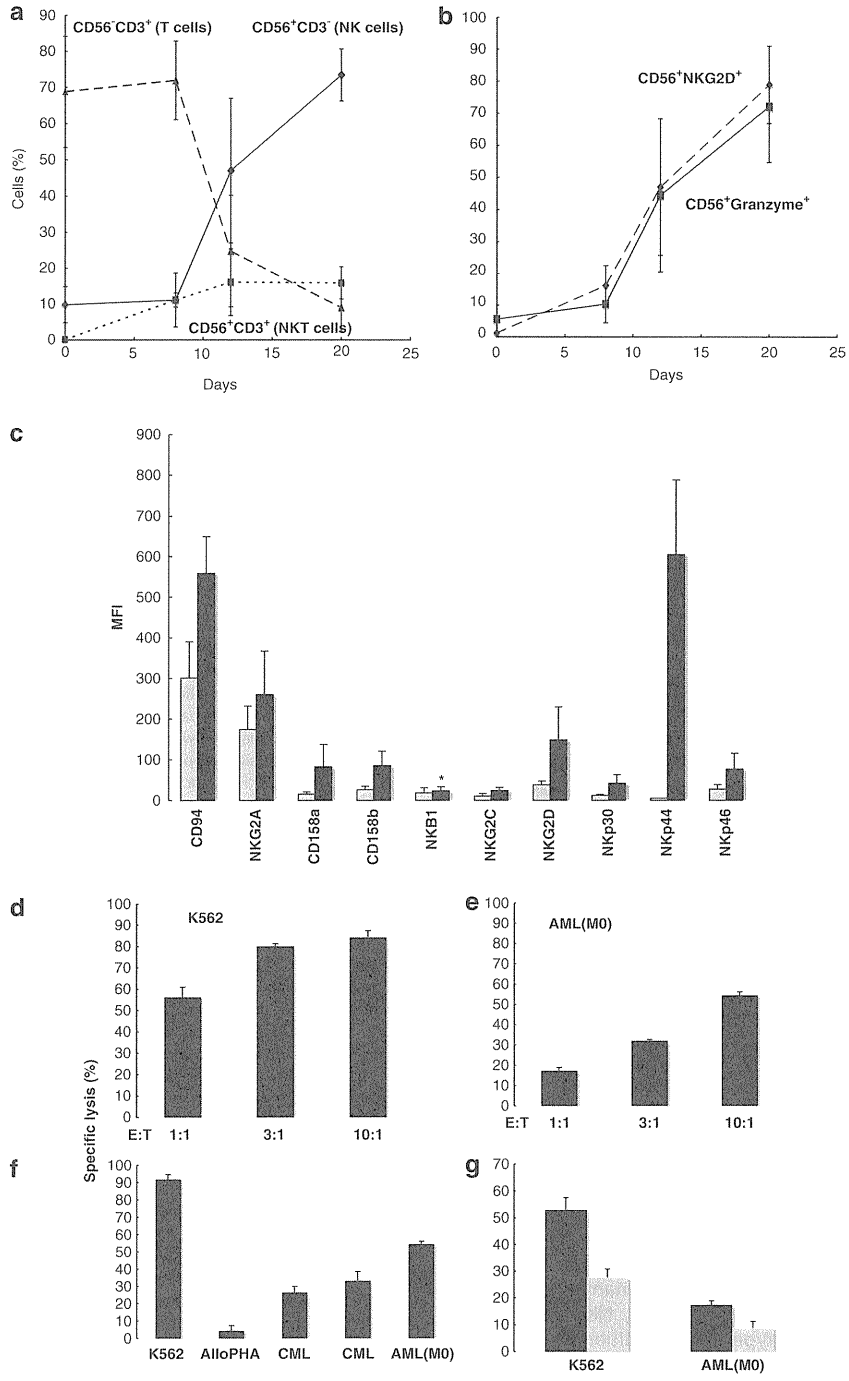

Figure 1. Time course profile of expanded cells during culture without feeder cells. $CD56^+CD3^-$ (NK cells), $CD56^-CD3^+$ (T cells) and $CD56^+CD3^+$ cells (NKT cells; **a**) and $CD56^+NKG2D^+$ cells and $CD56^+$granzyme$^+$ cells (**b**); bars indicate means ± s.d.s, $n = 5$. Mean fluorescence intensity (MFI) of NK receptors on $CD16^+CD56^+$ NK cells in CB before expansion (gray bars) and after expansion (black bars). There were significant differences between before and after expansion except for NKB1 *($P < 0.01$, $n = 10$; **c**). Cytolytic activities of expanded NK cells against K562 cells (**d**) and AML (M0) patient's leukemic cells (**e**). Data presented are means ± s.d.s (effector/target ratios of 1:1, 3:1 and 10:1). Cytolytic activities against K562 cells, allogeneic third-party PHA blasts and patients's leukemic cells from chronic myeloid leukemia and AML (M0; E:T ratio of 10:1; **f**). Inhibitory effect of anti-NKG2D monoclonal antibody (20 μg/ml, gray bars) against cytolytic activities of expanded NK cells (E:T ratio is 1:1; **g**).

a very high level of cytolytic activity against the K562 leukemic cell line, with specific lysis of more than 50% under the condition of an effector:target ratio (E:T ratio) of 1:1 and more than 80% under the condition of an E:T ratio of 3:1 (Figure 1d). Also, the expanded NK cells could attack patients' primary acute myeloid leukemia (AML; M0) and chronic myeloid leukemia (CP) leukemic cells, but could not attack allogeneic third-party PHA blasts (Figures 1e and f). Anti-NKG2D mAb (1D11, 20 μg/ml, Serotec, Oxford, UK)

suppressed the cytolytic activity against K562 cells and also patients' primary leukemic cells (Figure 1g). Therefore, the cytolytic activity of these expanded NK cells depended at least partially on NKG2D-activating receptor.

Allogeneic NK cells have been reported to have a strong GVL effect after haploidentical hematopoietic stem cell transplantation (HSCT) in patients with advanced AML, without causing GVHD.[3] Adoptive transfer of allogeneic NK cells may be a promising immunotherapy. However, expansion of NK cells seems to be difficult compared with expansion of T cells. About 20-fold expansion of NK cells was achieved by culture of PBMCs with cytokines for a short time and co-culture with K562 cells that had been transfected with, and expressed membrane-bound IL-15 and 4-1BBL. These expanded NK cells using artificial feeder cells had cytolytic activity against human AML cells and also pediatric solid tumors such as Ewing sarcoma and rhabdomyosarcoma. On the other hand, there have been several reports of NK cell expansion from PBMCs without using feeder cells. Alici *et al.*[10] reported the possibility of expanding NK cells without feeder cells from PBMCs of multiple myeloma patients with significant cytolytic activity against primary autologous multiple myeloma cells. It is more beneficial for clinical use if it is not necessary to use feeder cells for efficient expansion of NK cells *in vitro*. Also, Ayello *et al.*[11] reported a 20-fold expansion of NK cells from CB cells with depletion of adherent monocytes by *ex vivo* culture with IL-2, IL-7 and IL-12 for 7 days. Therefore, NK cells can be expanded from not only PBMCs, but also CB.

Clinical-scale NK cell purification has so far been performed by donor leukapheresis followed by CD3 depletion with or without CD56 enrichment. There have been several clinical reports on adoptive transfer of allogeneic NK cells for hematological malignancies. Passweg *et al.*[12] first reported the feasibility of allogeneic NK cell purification and infusion in five myeloid malignant patients after haploidentical HSCT. Miller *et al.*[13] reported that haploidentical NK cell infusions after cyclophosphamide and fludarabine treatment resulted in expansion of donor NK cells and induction of complete hematological remission in 5 of 19 AML patients with poor prognosis. A recent pilot study showed good results in pediatric patients who received haploidentical NK cells to consolidate chemotherapy for AML patients. Also, Nguyen *et al.*[14] reported a persistent and massive expansion of infused alloreactive NK cells in an AML patient who had relapsed after haploidentical HSCT. Yoon *et al.*[15] reported patients who underwent human leukocyte antigen-mismatched HSCT and subsequently received donor NK cells that were generated from CD34+ cells from donor leukapheresis products by *ex vivo* culture for more than 6 weeks (9.3×10^6 per kg, $CD122/CD56^+$ 64%, $CD3^+$ 1%). There were no signs of acute toxicity in 14 adult patients infused with these cells 6–7 weeks after transplantation, with one patient developing acute GVHD and five patients developing chronic GVHD. Therefore, clinical-grade allogeneic NK cell infusion is safe and feasible. However, many issues remain to be resolved, including selection of donor, NK cell selection and expansion procedure, type of conditioning regimen, survival and expansion of NK cells in the recipient after infusion, their localization and finally, the clinical effect of NK cell infusion.

In this study, we could obtain about 40×10^6 NK cells from 1×10^6 unmanipulated CB cells using tacrolimus and dalteparin sodium without feeder cells (more than 1000-fold expansion with more than 70% purity). Thus, alloreactive Killer cell immunoglobulin-like receptor (KIR)-mismatched expanded NK cells from CB can be used for adoptive NK cell immunotherapy to induce a strong GVL/tumor effect without severe GVHD for patients who do not have KIR-mismatched related donors.

CONFLICT OF INTEREST

A patent application for the composition for expanding NK cells and the use of them has been filed with Junji Tanaka as a sole inventor.

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ORIGINAL ARTICLE

Efficacy of folinic acid in preventing oral mucositis in allogeneic hematopoietic stem cell transplant patients receiving MTX as prophylaxis for GVHD

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As the safety of folinic acid administration and its efficacy for reducing the toxicity of MTX remain controversial, we assessed the effect of folinic acid administration after MTX treatment for GVHD prophylaxis on the incidence of oral mucositis and acute GVHD. We retrospectively analyzed data for 118 patients who had undergone allogeneic hematopoietic SCT and had received MTX for GVHD prophylaxis. Multivariate analysis showed that systemic folinic acid administration significantly reduced the incidence of severe oral mucositis (odds ratio (OR) = 0.13, 95% confidence interval (CI) 0.04–0.73, $P = 0.014$). There was also a tendency for a lower incidence of severe oral mucositis in patients who received folinic acid mouthwash (OR = 0.39, 95%CI 0.15–1.00, $P = 0.051$). No significant difference was observed in the incidence of acute GVHD between patients who received systemic folinic acid administration and those who did not ($P = 0.88$). Systemic folinic acid administration and mouthwash appear to be useful for reducing the incidence of severe oral mucositis in patients who have received allogeneic hematopoietic SCT using MTX as GVHD prophylaxis.

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Keywords: oral mucositis; folinic acid; SCT

Introduction

Oral mucositis is one of the most common complications associated with allogeneic hematopoietic SCT, occuring in 60–90% of patients who have received SCT.[1–3] Oral mucositis is associated with severe pain, which can lead to anorexia and dehydration. A large population of patients with severe oral mucositis require total parenteral nutrition and opioid analgesics.[4] Severe oral mucositis is associated with not only severe pain but also poor clinical and economic outcomes.[5]

Oral mucositis is caused mainly by the toxicity associated with chemotherapy and TBI as a conditioning regimen; however, it is also associated with the use of MTX for GVHD prophylaxis.[6,7] Although several studies have shown that folinic acid administration reduced the toxicity of MTX,[8–10] the efficacy and safety of folinic acid administration remain controversial. Ruutu *et al.*[11] reported that folinic acid was administered after MTX in 37 (45.7%) of 81 European Group for Blood and Marrow Transplantation (EBMT) centers, and Bhurani *et al.*[12] reported that folinic acid was administered after MTX in 8 (44.4%) of 8 centers in Australia and New Zealand. More than half of the centers surveyed in those studies did not use systemic folinic acid administration because of the lack of support for its efficacy or because of the risk of acute GVHD being induced by folinic acid.

Therefore, this study was performed to assess the effects of systemic folinic acid administration after MTX for GVHD prophylaxis on the incidence of oral mucositis and acute GVHD.

Patients and methods

We retrospectively analyzed data for 141 consecutive patients who had undergone allogeneic hematopoietic SCT and had received MTX for GVHD prophylaxis between March 2006 and December 2009 in Stem Cell Transplantation Center of Hokkaido University Hospital. We excluded seven patients whose data were insufficient. Furthermore, we excluded 16 patients who failed to achieve engraftment because we hypothesized that duration of neutropenia was a risk factor for the development of

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