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Clinical Research

Level of Vascular Endothelial Growth Factor Predicts Both Relapse and Nonrelapse Mortality after Allogeneic Hematopoietic Stem Cell Transplantation

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Although the prognostic significance of vascular endothelial growth factor (VEGF) has been researched extensively in patients with hematologic malignancies undergoing chemotherapy, its role in allogeneic hematopoietic stem cell transplantation (HSCT) requires further investigation. The present study evaluated the associations between VEGF level and relapse rate and early complications after HSCT. VEGF levels were analyzed in 91 consecutive patients before the start of conditioning, on day 0, on the day of engraftment, and on the day of diagnosis of veno-occlusive disease (VOD). Compared with a normal level, an elevated high VEGF-A level before conditioning was associated with an increased 2-year relapse rate (55% versus 24%, $P = .003$; hazard ratio [HR], 3.25; 95% confidence interval [CI], 1.49 to 7.08) and decreased event-free survival (20% versus 44%; $P = .022$; HR, 2.03; 95% CI, 1.11 to 3.72). No association was found between VEGF level and the incidence of acute GVHD ($P > .05$). In patients with VOD, VEGF-A level was elevated on day 0 and on the day of VOD diagnosis ($P < .05$). A low VEGF-A level on day 0 was associated with reduced nonrelapse mortality (14% versus 35%; $P = .048$; HR, 0.32; 95% CI, 0.10 to 0.99). Our results indicate that a high VEGF-A level before HSCT increases the risk of relapse, and a high level after conditioning is associated with increased risks of early complications and nonrelapse mortality.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is cytokine involved in angiogenesis [1]. The human VEGF gene family comprises 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor [2]. Considered one of the most potent angiogenic molecules, VEGF-A is involved in neovascularization in situations of reparation as well as tumor growth. Its significance in solid tumor growth is well established [3].

Recent studies have emphasized the role of VEGF-A in the progression of hematologic malignancies, with VEGF-A overexpression associated with altered morphology of bone marrow and increased vascularization of bone marrow in myeloproliferative disorders [4,5]. Elevated VEGF-A levels are also associated with worse chemotherapy outcomes in patients with acute myelogenous leukemia [6], chronic myelogenous leukemia [7], acute lymphoblastic leukemia [8], myelodysplastic syndrome [9], and various types of lymphoproliferation [10-12]. The significance of this negative prognostic factor in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) has not yet been established, however.

Apart from tumor-associated expression, VEGF-A expression is up-regulated in the presence of endothelial damage and has been extensively studied as an endothelial damage marker in cardiovascular disorders [13,14]. Although there is increasing interest in the role of endothelial dysfunction in the complications of HSCT [15,16], few studies to date have focused on VEGF-A. Several reports have suggested that VEGF-A may play a role in the development of acute graft-versus-host disease (GVHD) and that VEGF-A level is correlated with transplantation-related mortality [17-19]. The significance of VEGF-A in endothelial injury syndromes has not been evaluated in humans, but in animal models elevated levels were associated with increased vascular permeability and capillary leak, and thus may play a role in the pathogenesis of severe veno-occlusive disease (VOD) [20,21].

The primary goal of the present study was to test whether an elevated VEGF-A level before HSCT is associated with worse disease prognosis after allografting. A second goal was to investigate relationships among VEGF-A level, nonrelapse mortality (NRM), and major complications of HSCT, including acute GVHD and VOD.

PATIENTS AND METHODS

Patients

This study was based on the analysis of plasma samples and hospital records of 91 consecutive adult patients with a hematologic malignancy undergoing HSCT in R.M. Gorbacheva Memorial Institute of Children Hematology and Transplantation. Samples for the study were collected prospectively between 2010 and 2012. The study was approved by the Ethical Committee of I.P. Pavlov State Medical University, and informed consent was received from all patients for blood collection.

The distribution of disorders was 73% acute leukemia, 10% chronic myelogenous leukemia, 8% myelodysplastic syndrome, and 9% other hematologic malignancies. Median age was 38 years (range, 18 to 60 years), and median performance score and modified European Group for Blood and Marrow Transplantation risk score [22,23] were 1 (range, 0 to 3) and 4 (range, 1 to 6) respectively. The graft donor was related in 30% and unrelated in 70%. Pretransplantation conditioning was myeloablative in 24% and

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reduced-intensity in 76%. Myeloablative conditioning included 16 mg/kg oral busulfan and 120 mg/kg cyclophosphamide. Reduced-intensity conditioning was based on 180 mg/m^2 fludarabine and 8 mg/kg busulfan or 140 mg/m^2 melphalan in cases involving previous busulfan-based conditioning or anamnesis of neurologic disorders. Acute GVHD prophylaxis consisted of calcineurin inhibitor (tacrolimus in 81% of patients) and short-course methotrexate. Antilymphocyte globulin (Atgam; Pfizer, New York, NY) 60 mg/kg was used in unrelated transplants. All but 2 patients received a single allogeneic graft. Ten patients had undergone previous autologous HSCT. Detailed patient characteristics are given in Table 1. VOD prophylaxis with fixed-dose heparin was performed in all patients.

The staging and grading of acute GVHD were done using the modified Glucksberg consensus criteria [24] at the initiation of treatment. VOD was diagnosed clinically according to the modified Seattle criteria extended up to day +30 after HSCT, which required the presence of at least 2 of the following 3 clinical findings: jaundice with bilirubin >34 μmol/L, painful hepatomegaly, and fluid retention >5% of body weight. VOD was classified as severe in the presence of multiorgan failure.

Laboratory Methods

Venous blood was collected using EDTA anticoagulant from the central venous catheter before the start of conditioning, on the day of HSCT (day 0) before graft transfusion, on the day of engraftment, and on the day of VOD diagnosis. Blood samples were centrifuged for 15 minutes at 1000 × *g*, and aliquot plasma was stored in polypropylene tubes at −80°C until the day of the assay.

VEGF-A concentrations in plasma samples were measured by ELISA using a commercially available kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. The detection limit was 7.9 to 1000 pg/mL. Concentrations were determined without knowledge of clinical data.

Statistical Analysis

Data analysis was performed with SPSS version 17.0 (SPSS Inc, Chicago, IL). The chi-squared test and *t* test were used for univariate nonparametric and parametric analysis, respectively. VEGF levels in patients with VOD were analyzed using the Mann-Whitney *U* test. The diagnostic significance of VEGF in VOD was evaluated by receiver operating characteristic (ROC) curve analysis. The Kruskal-Wallis test was used to determine the effect of pre-transplantation therapy on VEGF level before conditioning. Kaplan-Meier survival analysis and the log-rank test [25,26] were used for univariate survival, transplantation-related mortality, and cumulative incidence of relapse comparisons. Multivariate analysis was performed using Cox regression [27]. Multivariate models were constructed using stepwise forward selection, using a *P* value ≤.01 (Bonferroni correction) to include variables in the model. The proportional-hazards assumption was tested for each variable individually; all variables met this assumption. VEGF cutoff levels for survival analysis were determined based on ROC curves. VEGF measurements at various time points were selected for Kaplan–Meier analysis and Cox regression if the ROC area under the curve (AUC) for that time point was ≥0.6.

Clinical data collection continued until day +100 after HSCT, after which patients were followed up for survival and relapse. The median duration of follow-up was 406 days (range, 135 to 730 days). NRM was defined as the cumulative incidence of death from the date of HSCT not related to the relapse and its subsequent treatment. Event-free survival (EFS) was defined as the time from the date of HSCT to the documented event (relapse or death).

Table 1
Patient and Transplantation Characteristics

Characteristic	Value
Number of patients	91
Males, n (%)	46 (50.5)
Females, n (%)	45 (49.5)
Age, y, median (range)	38 (15-60)
Performance status score, median (range)	1 (0-3)
Modified European Group for Blood and Marrow Transplantation risk score, median (range)	4 (1-6)
Diagnosis, n (%)	
Acute myelogenous leukemia	51 (56.0)
Acute lymphoblastic leukemia	15 (16.5)
Chronic myelogenous leukemia	9 (9.9)
Myelodysplastic syndrome	7 (7.7)
Multiple myeloma	3 (3.3)
Idiopathic myelofibrosis	3 (3.3)
Other malignancies	3 (3.3)
Disease status before HSCT, n (%)	
Remission	43 (47.3)
No remission	48 (52.7)
Previous autologous or allogeneic HSCT, n (%)	12 (13.2)
Cytogenetic risk, n (%)	
Low	1 (1.1)
Intermediate	38 (41.8)
High	27 (29.7)
Donor characteristics, n (%)	
Related	27 (29.7)
Unrelated	64 (70.3)
HLA-matched 10/10	76 (83.5)
Single HLA mismatch 9/10	15 (16.5)
Conditioning regimen, n (%)	
Myeloablative	22 (24.2)
Reduced-intensity	69 (75.8)
Transplant source, n (%)	
Bone marrow	26 (28.6)
Mobilized peripheral blood stem cells	62 (68.1)
Both	3 (3.3)
CD34$^+$ cell dose, × 10^6/kg, mean ± SD	5.2 ± 2.0

RESULTS

We analyzed VEGF concentrations in 91 consecutive patients undergoing HSCT. Engraftment was achieved in 90% of patients, and the other 10% had primary graft failure or progressive disease. In the entire study group, 2-year overall survival (OS) was 51%, 2-year EFS was 38.5%, 2-year NRM was 30.8%, and the 2-year cumulative incidence of relapse was 31%. VOD was diagnosed in 13 patients (14%), with severe VOD in 10%. The median time to VOD diagnosis was 13 days (range, 6 to 21 days) after HSCT, and the median interval between VOD diagnosis and engraftment was 5 days. Acute GVHD grade I-IV was diagnosed in 54.4% of the patients; grade III-IV, in 20.9%.

VEGF-A Concentrations

The mean VEGF-A concentration was 177 ± 799 pg/mL before conditioning, 147 ± 660 pg/mL on day 0, and 171 ± 781 pg/mL on engraftment. Concentrations were below the level of quantification (<7.8 pg/mL) in 24.2% before conditioning, in 31.9% on day 0, and in 27.5% on engraftment. The widely variable results can be attributed mainly to 6 patients (3 with chronic myelogenous leukemia, 1 with acute lymphoblastic leukemia, 1 with acute myelogenous leukemia, and 1 with acute plasmoblastic leukemia) with concentrations above the upper limit of quantification at 2 or 3 of the 3 time points and with samples tested in dilution. All 6 of these patients had either no hematologic remission or measurable minimal residual disease at the time of HSCT, and thus the high concentrations in these patients were attributed to tumor-associated production.

VEGF-A Level and Relapse Rate

Patients who underwent HSCT without hematologic remission had higher preconditioning and day 0 VEGF-A levels compared with those undergoing HSCT while in remission (349 ± 1158 versus 29 ± 57 pg/mL [$P = .11$] and 263 ± 926 versus 68 ± 377 [$P = .004$], respectively), although the difference at the preconditioning time point was not statistically significant. Only VEGF level before the start of conditioning had the required statistical power to predict relapse. The AUC values for VEGF conditioning were 0.61 before conditioning, 0.51 on day 0, and 0.49 on engraftment. For subsequent relapse and survival analysis, patients were divided into 2 groups according to the predetermined VEGF cutoff level of 37 pg/mL. Patients with a VEGF-A concentration >37 pg/mL had an elevated cumulative relapse rate (55%

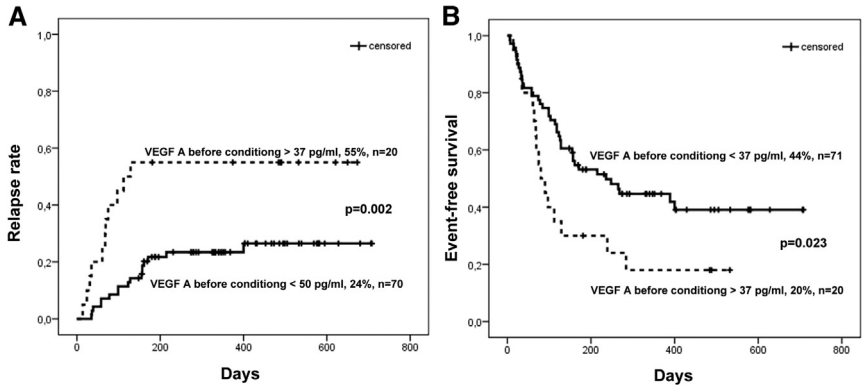

Figure 1. Two-year cumulative incidence of relapse (A) and 2-year EFS (B) according to VEGF-A level before conditioning.

versus 24%; $P = .002$) and, consequently, a decreased 2-year EFS (20% versus 44%; $P = .023$) compared with those with a level below the cutoff (Figure 1). Surprisingly, OS did not differ significantly in the 2 groups (61% versus 49%; $P = .334$), because the majority of patients in the high VEGF group responded to salvage therapy after relapse. In multivariate analysis, VEGF-A concentration >37 pg/mL before the start of conditioning remained a significant independent risk factor for relapse (HR, 3.33; 95% CI, 1.15 to 7.32; $P = .003$) and decreased EFS (HR, 2.03; 95% CI, 1.11 to 3.72; $P = .022$) (Table 2).

Similar results were observed for the less-heterogenous group of acute leukemia patients with a pretransplantation VEGF-A level >37 pg/mL. Owing to the small number of patients, we found only a statistical trend toward an increased relapse rate (46% versus 25%; $P = .07$), although the difference in EFS remained significant (23% versus 51%; $P = .039$).

We performed an additional analysis to exclude the effect of pretransplantation chemotherapy on VEGF-A level before conditioning. Time from diagnosis to HSCT ($P = .83$), number of previous courses of chemotherapy ($P = .12$), number of courses of high-dose chemotherapy ($P = .82$), previous HSCT ($P = .11$), and time from the last course of chemotherapy ($P = .28$) had no significant effect on VEGF-A level.

VEGF-A Level and Early Complications of HSCT

We found no statistically significant differences in VEGF levels before conditioning, on day 0, and on engraftment between patients with and those without acute GVHD grade I-IV and grade III-IV ($P > .05$). This study had an additional focus on VOD, considering that this complication of HSCT is known to be associated with significant endothelial damage and there was an additional blood collection time point on the day of VOD diagnosis. Because VOD was diagnosed on different days after HSCT between day 0 and engraftment, these latter 2 time points were used for comparison in the non-VOD group. VEGF levels before conditioning and on engraftment were not significantly different in the 2 groups (42 ± 64 pg/mL versus 53 ± 154 pg/mL [$P = .719$] and 26 ± 28 pg/mL versus 23 ± 35 pg/mL; $P = .718$], respectively). However, in the VOD group, higher VEGF-A levels were observed on day 0 (52 ± 55 pg/mL versus 33 ± 120 pg/mL; $P = .045$) and on the day of VOD diagnosis compared with those seen in the non-VOD group on day 0 (128 ± 147 pg/mL versus 33 ± 120 pg/mL; $P = .003$) and the day of engraftment (128 ± 147 pg/mL versus 23 ± 35 pg/mL; $P = .008$) (Figure 2).

To evaluate the predictive significance of day 0 VEGF level on the incidence of VOD, we divided the patients into 2 groups based on a VEGF-A cutoff level of <7.8 pg/mL that was subsequently found to be highly specific for overall NRM. Patients in the low-VEGF group demonstrated only a trend toward reduced risk of VOD (7% versus 25%; $P = .126$), but

Table 2
Univariate and Multivariate Analysis of Factors Effecting Cumulative Relapse Rate, NRM, and EFS

Factor	Univariate *P* Value	Multivariate *P* Value	HR (95% CI)
Cumulative relapse rate			
Unrelated versus related donor	.007	.039	0.46 (0.21-0.96)
Active disease at the time of HSCT	.094		
Myeloablative versus reduced-intensity conditioning	.156		
Acute GVHD grade I-IV	.026	.046	0.44 (0.20-0.99)
Chronic GVHD	.021	.070	
VEGF-A >37 pg/mL before conditioning	.002	.003	3.25 (1.49-7.08)
NRM			
Active disease at the time of HSCT	<.001	.001	4.63 (1.83-11.69)
Age	.044	.019	0.96 (0.93-0.99)
Previous HSCT	.002	.029	3.18 (1.29-8.96)
Unrelated versus related donor	.20		
Myeloablative versus reduced-intensity conditioning	.43		
Acute GVHD grade III-IV	<.001	.006	3.96 (1.48-10.58)
VOD	<.001	.024	3.33 (1.69-9.47)
VEGF-A <7.8 pg/mL on day 0	.046	.048	0.32 (0.10-0.99)
EFS			
Active disease at the time of HSCT	<.001	<.001	3.70 (1.87-7.35)
Age	.13		
Previous HSCT	<.001	.004	3.06 (1.44-6.49)
Unrelated versus related donor	.28		
Myeloablative versus reduced-intensity conditioning	.44		
Acute GVHD grade I-IV	.018	.051	
Chronic GVHD	<.001	<.001	0.26 (0.13-0.54)
VOD	<.001	.007	2.9 (1.34-6.28)
VEGF-A >37 pg/mL before conditioning	.023	.022	2.03 (1.11-3.72)
VEGF-A <7.8 pg/mL on day 0	.55		

HR >1 indicates increased risk of relapse and NRM, and decreased EFS.

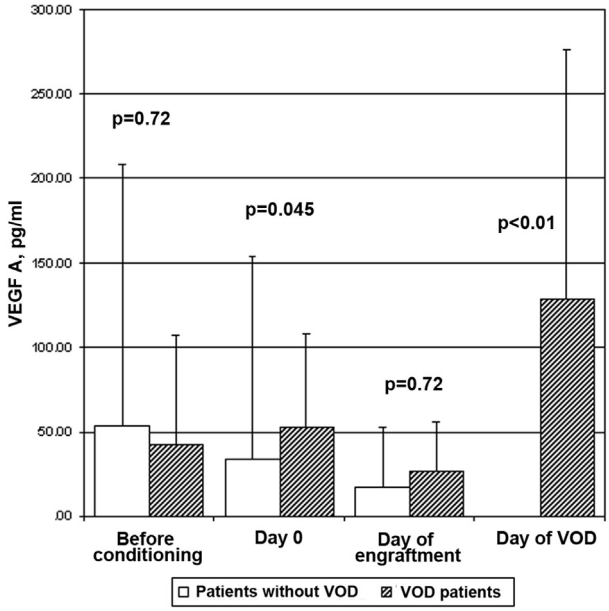

Figure 2. Level of VEGF-A in patients with and without VOD.

a statistically significant reduction in the risk of severe VOD (0% versus 19%; $P = .024$). Despite significant elevated VEGF levels in the patients with VOD, the utility of VEGF-A as prognostic marker (AUC = 0.74) or a diagnostic marker (AUC = 0.55) of VOD was relatively low, with sensitivity of 67% and 62% and specificity of 79% and 58%, respectively.

VEGF-A Level and NRM

Only VEGF-A level on day 0 was predictive for NRM (AUC = 0.67), whereas levels before conditioning (AUC = 0.46) and on engraftment (AUC = 0.57) lacked sufficient predictive power. The VEGF cutoff level on day 0 with the greatest sensitivity and specificity for NRM was below the limit of quantification of 7.8 pg/mL. Patients with a VEGF-A level <7.8 pg/mL had a significantly lower NRM on day 0 (14% versus 35%; $P = .046$) (Figure 3). In a multivariate analysis of factors predicting NRM, VEGF-A level below the limit of quantification was an independent factor associated with reduced mortality (HR, 0.32; 95% CI, 0.10 to 0.99; $P = .048$) (Table 2). Our analysis of underlying causes of NRM in the low-VEGF and high-VEGF groups could not determine the reason for the observed differences in mortality. Mortality from infectious complications ($P = .77$), acute GVHD ($P = .94$), graft failure ($P = .39$), and vascular complications, including VOD ($P = .79$), differed in the 2 groups on day 0.

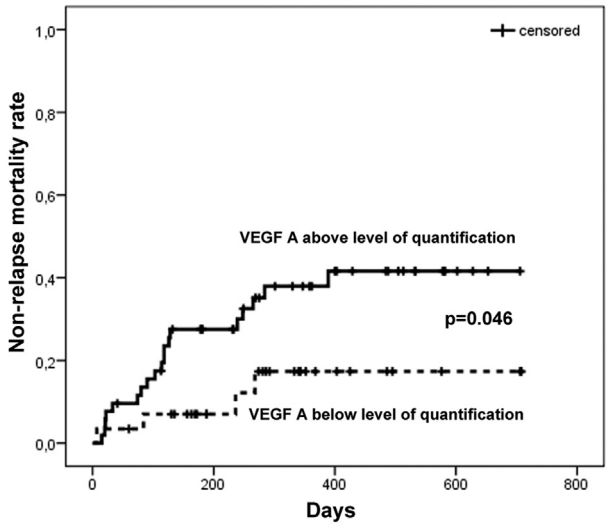

Figure 3. Two-year NRM according to VEGF-A level on the day of HSCT.

DISCUSSION

This study of VEGF-A measured at different time points surrounding HSCT demonstrates the dual role of VEGF-A. Its overexpression before conditioning was associated with increased risk of relapse and might represent a mechanism of tumor resistance, given that none of the pretransplantation factors were found to affect VEGF-A levels. On the other hand, the observed association between VEGF-A level after conditioning and NRM and VOD confirms VEGF-A's significance as an endothelial damage marker in the setting of HSCT.

Tumor-associated production of VEGF-A as a possible mechanism of resistance to chemotherapy has been studied extensively in various hematologic neoplasms [6-12], but to the best of our knowledge, this is the first study to confirm the negative prognostic effect of a high VEGF-A level before conditioning on HSCT outcomes. Another important finding of this research is that despite an increased rate of early relapse, there were no differences in overall survival in high VEGF and low VEGF groups of patients. Although the follow-up was relatively short, and longer observational periods might have revealed differences, successful post-transplantation salvage chemotherapy and donor lymphocyte infusion in at least some patients in the high–VEGF-A group demonstrates the potential utility of the graft-versus-leukemia effect to overcome this factor in resistance. Allogeneic HSCT is considered a treatment of choice for high-risk disease in patients with acute leukemias, myelodysplastic syndrome, chronic myelogenous leukemia, and lymphomas [28-31]. Given that standard therapy outcomes for these malignancies have been reported to be negatively influenced by VEGF-A overexpression, more studies are needed to determine whether patients with high VEGF-A levels should be candidates for HSCT.

Interpreting the data on VEGF-A as an endothelial damage marker requires an understanding of the mechanisms and factors regulating its level in plasma. VEGF-A is secreted by a wide variety of cells, including endothelial and blood mononuclear cells. It is eliminated via cleavage with serum proteases and binding to either soluble or cellular VEGF receptors. However, a large proportion of VEGF is bound to extracellular matrix (ECM) heparin sulfate proteoglycans. In response to endothelial and tissue damage, ECM-bound VEGF is cleaved from proteoglycan complexes and released into circulation to facilitate reparation. The important factor is that serum protease activity in terms of VEGF elimination is insufficient to decrease levels in situations of extensive release from ECM [32-34], resulting in elevated levels of circulating VEGF in cases of substantial endothelial damage, such as a conditioning regimen. Another important factor is that VEGF-A is bound to heparin. Several studies have indicated that administration of heparin results in decreased serum VEGF levels, which may be falsely interpreted [35,36]. In the present study, all patients received a fixed-dose continuous infusion of heparin and all blood collections were done at the steady-state level, and thus

there should be no interpatient or intrapatient variability of VEGF concentrations related to this factor.

Observed differences in NRM according to VEGF-A level on day 0 confirm the significance of endothelial damage by conditioning on the outcome of HSCT. Levels of another molecule involved in angiogenesis, angiopoietin-2, have been correlated with NRM as well [37,38]. In this study, the statistical analysis of VEGF levels allowed to distinguish only patients with low risk of NRM. This could be explained by possible residual tumor-associated secretion on day 0 in patients with VEGF-A–expressing neoplasms, which interfered in the analysis and did not allow to distinguish the high-risk group for NRM, but we were not able to confirm this hypothesis in our research. In addition, owing to our limited number of patients, we could not analyze the differences in the major causes of NRM, including infectious complications, acute GVHD, graft failure, and vascular complications. Thus, the question of what complications are associated with early mortality after HSCT in patients with significant endothelial damage may be a subject for future research.

Previous studies of VEGF-A have demonstrated controversial results regarding the association between VEGF-A level and acute GVHD. Lunn et al. [18] reported higher VEGF-A levels in patients with acute GVHD; in contrast, Min et al. [17] found that low VEGF-A levels on days +7 to +14 after HSCT was a risk factor for severe acute GVHD. This was confirmed in a laboratory mouse model in which blockage of VEGF-A resulted in an increased rate of GVHD [39]. In the study of Luft et al. [37], as well as the present study, no association between VEGF-A level and GVHD was found. These discrepancies in the literature may be explained by different study designs, sample collection at different time points surrounding HSCT, and the use of plasma or serum for analysis. Thus, selection of representative time points for VEGF measurement is crucial for further research in this field.

The results of the present study regarding VOD confirm the presence of extensive endothelial cell injury during the course of this HSCT complication [40-42]. The research protocol included 1 additional blood collection point on the day of VOD diagnosis. This time point was compared with VEGF levels on day 0 and the day of engraftment in patients who did not develop VOD. This applies to some limitations of our study, because the assay was performed on different days after HSCT in the patients with VOD, but, as reported previously, peaks of endothelial damage are observed on the day 0 and on engraftment [43], justifying the use of day 0 and day of engraftment time points in non-VOD patients for comparison. Preclinical research has linked high circulating VEGF-A concentrations with capillary leak and lung edema [20,21]; thus, we had a preliminary hypothesis that capillary leak in severe VOD might be related, at least in part, to elevated VEGF levels. Although significantly elevated VEGF levels were detected in the patients with VOD, concentrations up to 10-fold higher were observed in patients with tumor-associated preconditioning VEGF elevation with no clinical evidence of capillary leak syndrome. Thus, it is more likely that other cytokines, such as TNF-α and IL-2, are involved in the pathogenesis of severe VOD [44-46].

In conclusion, VEGF-A is an informative endothelial marker that can be used to predict NRM and VOD after HSCT. It also plays an important role in the progression of hematologic malignancies and may be a promising target for future therapies. Numerous anti-VEGF experimental agents for treating hematologic tumors are currently under evaluation [47,48], but these should be used with caution in the setting of HSCT owing to the possible protective effects of VEGF against early post-transplantation complications.

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