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Circulating Angiogenic Factors Associated with Response and Survival in Patients with Acute Graft-versus-Host Disease: Results from Blood and Marrow Transplant Clinical Trials Network 0302 and 0802

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A B S T R A C T

Circulating angiogenic factors (AF) reflect tissue healing capacity, although some AF can also contribute to inflammation and are indicative of endothelial dysfunction. The AF milieu in acute graft-versus-host disease (aGVHD) has not been broadly characterized. We hypothesized that patients with abundant AF involved in repair/regeneration versus those mediating damage/inflammation would have improved outcomes. Circulating AF known predominantly for repair/regeneration (epidermal growth factor [EGF], fibroblast growth factor-1 and -2, heparin binding–EGF–like growth factor, and vascular endothelial growth factor-A [VEGF-A], -C, and -D) and for damage/inflammation (angiopoietin-2, endothelin-1, soluble endoglin [sEng], follistatin [FS], leptin, and placental growth factor [PlGF]) were measured in a discovery set of hematopoietic cell recipients with grade III and IV aGVHD and compared with controls, then validated in 2 aGVHD cohorts enrolled in Blood and Marrow Transplant Clinical Trials Network (BMT CTN) trials 0302 (n = 105, serum) and 0802 (n = 158, plasma) versus controls without aGVHD (n = 53, serum). Levels of EGF and VEGF-A were lower than in controls at the onset of aGVHD in both trials and higher with complete response to first-line aGVHD therapy in CTN 0802. FS and PlGF were elevated in aGVHD measured in either serum or plasma. At day 28 after initial aGVHD therapy, elevated FS was an independent negative prognostic factor for survival in both cohorts (hazard ratio, 9.3 in CTN 0302; 2.8 in CTN 0802). These data suggest that circulating AF are associated with clinical outcomes after aGVHD and, thus, may contribute to both pathogenesis and recovery.

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BACKGROUND

In the United States, nearly 7000 patients undergo allogeneic hematopoietic cell transplantation (HCT) annually in an effort to cure hematologic malignancies and other bone marrow disorders. Approximately 50% of these patients will experience acute graft-versus-host disease (aGVHD), a complication in which cells from the immunocompetent donor graft attack the recipient's organs and tissues [1]. Only one half of aGVHD patients achieve a durable response to first-line therapy with corticosteroids [2] and many others develop severe infections resulting from the immunocompromised state and impairment of skin and mucosal barrier integrity. As a result, many patients with severe aGVHD are at significant risk of death [3]. Identification of novel treatment approaches that can alleviate inflammation, spare infectious immunity, and promote healing is critical for improving patient outcomes.

A host vascular proliferative response accompanying aGVHD was first described by Brent and Medawar in the

1960s [4], although it was not clear whether the angiogenic response in aGVHD could improve outcomes by facilitating healing or if it was detrimental to outcomes by contributing to inflammation. Recently, mechanisms underlying this critical observation have been suggested using rodent models. Specifically, vasculogenesis accompanies aGVHD with a concomitant increase in alpha-v integrin expression on endothelial cells in tissues targeted by aGVHD, a pathologic reaction that can be inhibited by a negative regulator of neovascularization, micro RNA-100, and by the alpha-v integrin inhibitor cilengitide [5,6]. Despite these advances, tools to therapeutically target neovascularization in human aGVHD are lacking. Recent literature suggests that angiogenic factors (AF), soluble mediators that support the development of new blood vessels, may contribute to favorable outcomes by providing trophic factors for wound healing after injury. This principle has been demonstrated in the autoimmune inflammatory bowel disease (IBD) setting with epidermal growth factor (EGF), an epithelial and endothelial mitogen that enhances angiogenic responses in tissues [7,8]. In IBD, circulating EGF levels have been shown to be low [9], and supplementation has induced remission in a randomized trial [10].

Studies of such AF capable of repairing host tissues and their associations with aGVHD outcomes are emerging. Patients with single nucleotide polymorphisms associated with increased production of vascular endothelial growth factor (VEGF) have a reduced incidence of grade II to IV aGVHD, including gastrointestinal aGVHD [11]. In addition, single nucleotide polymorphisms within the gene encoding thrombomodulin, a constitutively expressed endothelial factor that enhances angiogenesis [12], are associated with survival after onset of aGVHD [13]. These genetic studies suggest that variations in the patient's capacity to repair tissues after both the damage from the conditioning regimen and at the onset of aGVHD are clinically relevant. However, not all reports have consistently shown a beneficial role for AF in aGVHD. High circulating levels of VEGF have been shown to be protective in some aGVHD studies [14,15] but not in others [16,17]. It is possible that other factors contributing to the angiogenic milieu may be responsible for the discrepant results. For example, angiopoietin-2 (Ang2) is an AF that can have either proangiogenic or antiangiogenic function, depending upon the context [18]. In allogeneic HCT, elevated levels of Ang2 are reported to reflect endothelial cell activation/dysfunction and are associated with a poor prognosis in aGVHD [17,19,20].

Given these disparate associations of AF with outcomes in aGVHD, we sought to more broadly characterize the angiogenic milieu in aGVHD and determine the association of individual factors with clinical outcomes. Furthermore, we compared serum versus plasma levels of AF, because of the potential for variability contributed by factor release by activated platelets, a nontrivial matter for future studies of AF in human disease. Thirteen AF were first tested in a pilot study, followed by the validation of 6 AF using samples from 2 multicenter aGVHD treatment trials, Blood and Marrow Transplant Clinical Trials Network (BMT CTN) 0302 [21], a randomized 4-arm phase II clinical trial for patients with newly diagnosed aGVHD where serum was collected, and BMT CTN 0802 [22], a randomized phase III study of the addition of mycophenolate mofetil versus placebo to corticosteroids in patients with newly diagnosed aGVHD, where plasma was collected. Overall, we hypothesized that patients with abundant circulating AF involved in repair/regeneration would have improved outcomes compared with those with higher levels of AF involved in damage/inflammation.

PATIENTS AND METHODS
Discovery Cohort

We measured circulating levels of EGF, fibroblast growth factor (FGF)-1, FGF-2, heparin binding−EGF−like growth factor, VEGF-A, -C, and -D, Ang2, endothelin-1, endoglin (sEng), follistatin (FS), leptin, and placental growth factor (PlGF) in HCT recipients at onset of grade III and IV aGVHD (patient discovery set, n = 17) and compared them with recipients without aGVHD at 3 months after HCT (n = 17) and healthy stem cell donors (n = 16). Therefore, samples from both HCT patients without aGVHD and healthy donors were tested as controls. Samples were analyzed by MILLIPLEX (Millipore, Billerica, MA) magnetic bead array and performed in duplicate. For the pilot study, plasma samples were collected in sodium heparin tubes, except for 13 of the aGVHD specimens, which were serum. The mean coefficient of variation for the analytes ranged from 1.9% to 7.5%.

Validation Cohorts

AF demonstrating statistically significant <.5-fold or >1.5-fold differences from controls in the pilot study were selected for validation in samples from 2 multicenter treatment trials for initial therapy of aGVHD: BMT CTN 0302 (n = 105) and BMT CTN 0802 (n = 158). Serum (0302) or plasma (0802) samples obtained at aGVHD onset and at day 28 after GVHD treatment were selected for study. All patients with available aGVHD onset and day 28 post-aGVHD therapy samples were included in this study. Samples from aGVHD onset in these 2 trials were further compared with serum samples from a control cohort of HCT patients without acute or chronic GVHD (n = 53). These control samples were collected 3 months after HCT in University of Minnesota allogeneic HCT recipients, a time point consistent with the pilot study. Samples were acquired after obtaining informed consent and approval from the University of Minnesota institutional review board and in accordance with the Declaration of Helsinki.

Statistical Analysis

Statistical comparisons across categorical variables were completed using chi-square tests. Differences in continuous variables across categories were completed using Kruskal-Wallis tests for nonparametric data [23]. Differences between AF levels in onset and day 28 samples were determined using Wilcoxon signed rank tests [24]. Kaplan-Meier estimates were used to determine the probability of 2-year survival, with differences between curves determined using log-rank tests [25]. Cox regression was used to determine the independent effects of clinical factors and angiogenic biomarkers on 6-month and overall survival [26]. Correlations between continuous variables were determined by Spearman's rank correlation [27]. Receiver operating characteristic (ROC) curves were generated to determine the value of the tested AF in discriminating aGVHD from no aGVHD. Statistical analyses were performed using JMP 10.0.0 (SAS Institute, Cary, NC).
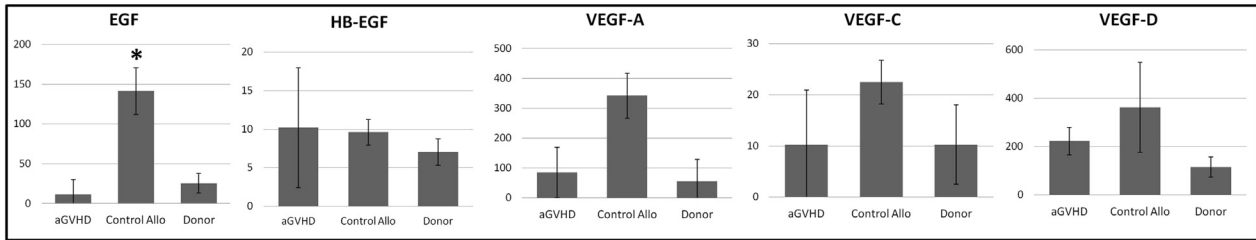
RESULTS
Discovery Set

In the discovery cohort, levels of multiple AF differed at aGVHD onset compared with levels for controls (Figure 1). Specifically, among the repair and regeneration factors, the median EGF level was 10-fold lower in aGVHD patients compared with those of allogeneic HCT patients without aGVHD. Similar to EGF, the median VEGF-A level was 5-fold lower in patients with aGVHD compared with that of control allogeneic HCT patients and 1.5-fold higher than normal donors (overall P value of .07). Based on these data and its importance in angiogenesis, VEGF-A was retained for validation studies. Four AF associated with damage/inflammation were significantly elevated in aGVHD patients compared with the levels in healthy donors: Ang2, sEng, FS, and PlGF. Too few patients had detectable levels of endothelin-1, FGF-1, and FGF-2 for statistical comparison (not shown). Using the pilot study data, 6 AF were selected for validation: FS, EGF, VEGF-A, Ang2, sEng, and PlGF.

Validation Cohorts

Characteristics of the patients in the validation cohorts are detailed in Table 1. The median age was 6 to 8 years

Repair and Regeneration
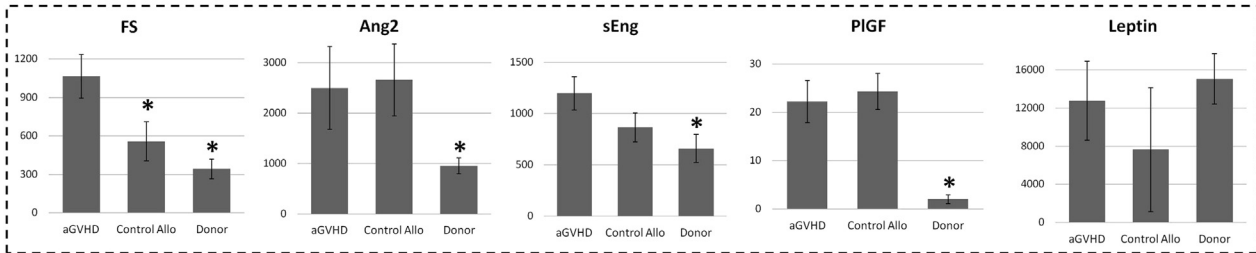

Damage and Inflammation

Figure 1. Discovery set. All results are pg/mL. *Denotes controls versus aGVHD.

higher in the control cohort (median, 58 years versus 50 and 52 years, $P = .01$), and consequently more patients in the control cohort underwent reduced-intensity conditioning as opposed to myeloablative HCT. Patients in the control cohort had a higher proportion with umbilical cord blood as the stem cell source ($P < .001$), reflecting institutional practices in unrelated donor HCT. The majority of patients in the BMT CTN trials had mild (grade I and II) aGVHD. There was no difference in the proportion of clinical responses (complete plus partial responses) to first-line therapy at day 28 between the 2 BMT CTN studies (70.5% and 74.7%, $P = .19$).

Levels of 5 of the 6 AF studied showed significant differences at onset of aGVHD compared with levels of control

recipients without aGVHD (Figure 2, Supplemental Table S1). Initially, we compared serum levels (from CTN 0302) and plasma (from CTN 0802) AF to serum controls. VEGF-A and EGF were significantly lower in patients with aGVHD than they were in controls, similar to findings in the pilot study. Additionally, plasma levels of these factors were even lower than the serum levels. The serum levels of VEGF-A in CTN 0302 were not statistically different than in controls, whereas EGF levels were significantly lower in both serum and plasma from aGVHD patients. Ang2 serum or plasma levels were similar to those in controls. Levels of both serum and plasma FS and PlGF were significantly elevated in patients with onset aGVHD compared with those in control

Table 1
Patient Characteristics

Variable	BMT CTN 0302		BMT CTN 0802		Control Allo HCT		P Value
	(n = 105)	%	(n = 158)	%	(n = 53)	%	
Age, median (range)	50.3 (7.5-69.9)		52.4 (9.1-76.3)		58 (19-72)		.01
Donor type							<.001
MSD	42	40.0	50	31.6	32	60.4	
Matched URD	37	35.2	84	53.2	1	1.9	
Mismatched URD	2	1.9	2	1.3	0	0.0	
Other related	7	6.7	11	7.0	0	0.0	
UCB	16	15.2	2	1.3	20	37.7	
Missing	1	1.0	9	5.7	0	0.0	
Conditioning							<.001
MA	66	62.9	97	61.4	13	24.5	
RIC	38	36.2	52	32.9	40	75.5	
Missing	1	1.0	9	5.7	0	0.0	
Days to aGVHD, median (range)	33 (13-146)		34 (11-165)		N/A		.90
Initial GVHD grade					N/A		.48
I	12	11.4	20	12.7			
II	64	61.0	90	57.0			
III	26	24.8	29	18.4			
IV	2	1.9	5	3.2			
Missing	1	1.0	14	8.9			
Day 28 response					N/A		.19
CR	54	51.4	76	48.1			
PR	20	19.0	42	26.6			
NR	29	27.6	31	19.6			
Missing	2	1.9	9	5.7			

Allo HCT indicates allogeneic hematopoietic cell transplantation; MSD, matched sibling donors; URD, unrelated donor; UCB, umbilical cord blood; MA, myeloablative; RIC, reduced-intensity conditioning; N/A, not applicable.
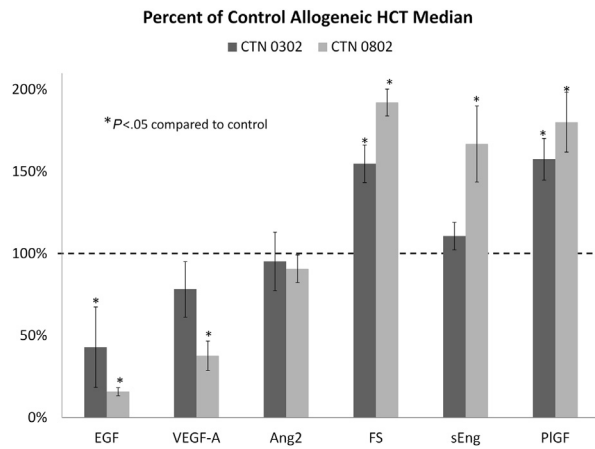
Figure 2. Circulating AF at onset of aGVHD in 2 aGVHD treatment trials versus allogeneic HCT controls.

allogeneic HCT patients, but the increase was greater in plasma. Plasma endoglin levels (from CTN 0802) were significantly elevated over control serum. This was not seen in aGVHD serum (from CTN 0302).

ROC analyses of data pooled from BMT CTN 0302 and 0802 revealed that 4 of the 6 AF levels could discriminate aGVHD presence versus its absence: EGF, VEGF-A, FS, and sEng (Figure 3). The highest sensitivity was observed with EGF at a level of 70.9 pg/mL, demonstrating a sensitivity of 80%, albeit with a lower specificity at 35%, and an area under

the curve (AUC) of .76 ($P = .03$). sEng at a level of 1,485.7 pg/mL had the highest discrimination with, a sensitivity of 42%, a high specificity of 89%, with an AUC of .63 ($P = .002$). ROC curves generated using only serum samples from CTN 0302 versus serum controls, only VEGF-A, with an AUC of .6 ($P = .046$) could discriminate between aGVHD and controls without GVHD. In non-GVHD controls, donor type did influence AF levels (not shown). Reduced-intensity conditioning was associated with lower Ang2 levels than myeloablative conditioning (median, 3407 pg/mL versus 5235 pg/mL, $P = .038$). No other clinical variables were associated with AF levels in controls and thus did not influence the ROC analysis.

Association of AF with Patient and Donor Factors

AF levels were not associated with time to aGVHD onset, severity, or organ tropism consistently in either aGVHD trial (Supplemental Tables S2-S4). EGF levels were significantly lower in recipients of myeloablative conditioning compared with recipients of reduced-intensity conditioning in CTN 0302 (38 versus 89 pg/mL, $P = .005$) but not in CTN 0802 ($P = .40$). In CTN 0802, recipient age was associated with a trend toward higher onset FS levels ($P = .07$) as well as a trend toward lower day 28 EGF levels ($P = .06$). PlGF levels were 1.5-fold higher at the onset of aGVHD in unrelated donors compared with in HLA-matched siblings in both trials (CTN 0302, $P = .03$; CTN 0802, $P = .007$). No other clinical factors were associated with levels of these 6 AF at onset or day 28 after initial therapy for aGVHD.
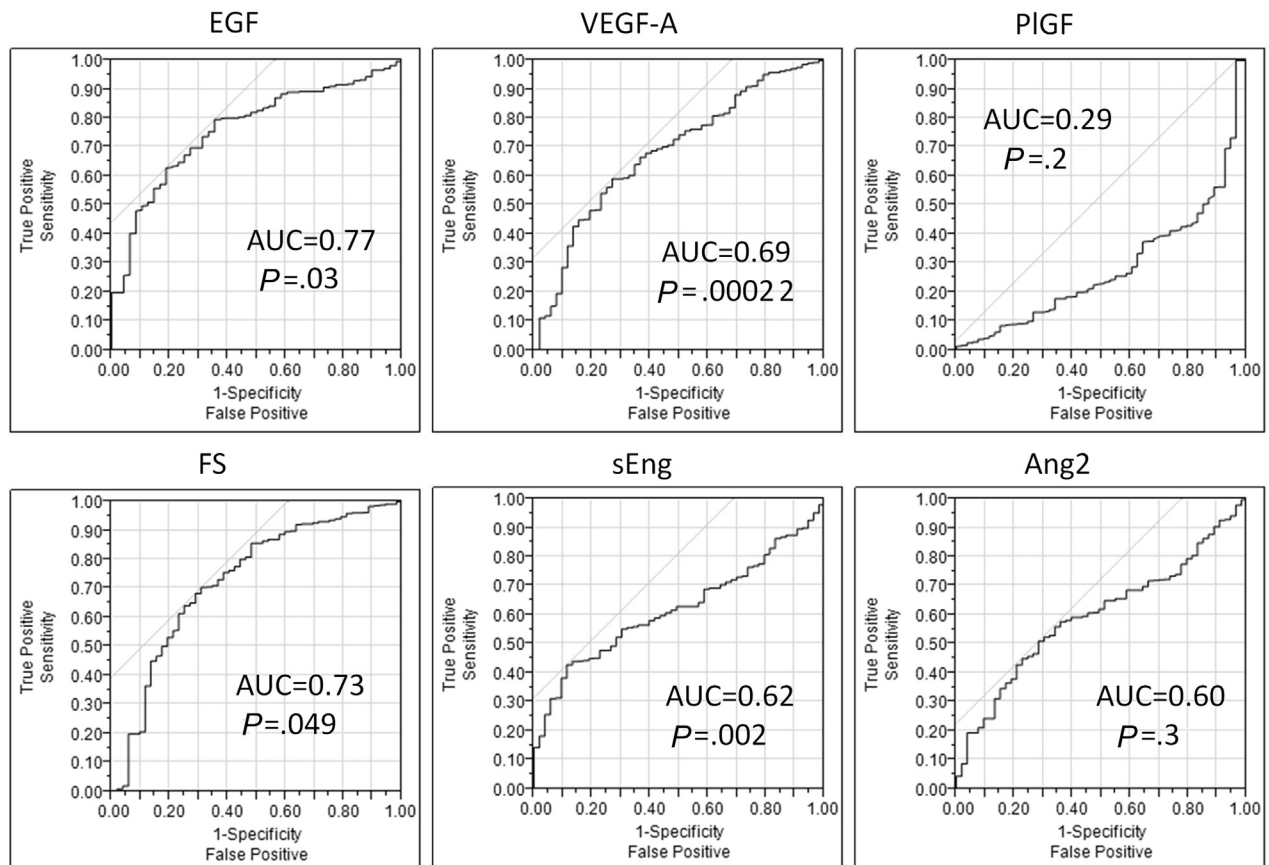
Figure 3. Receiver operating characteristic (ROC) curves for 6 AF discriminating between the presence or absence of aGVHD.
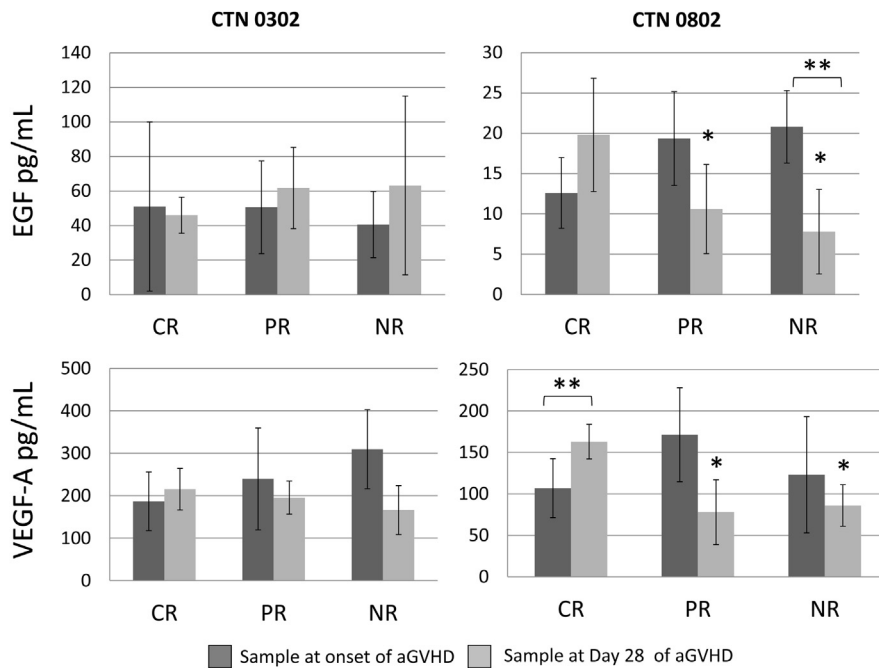
Figure 4. Association of circulating EGF and VEGF-A at onset of aGVHD and at day 28 after initial aGVHD therapy and clinical response. *Denotes $P < .05$ compared to complete response. **Denotes $P < .05$ day 28 sample compared to the sample from the onset of aGVHD.

Association of AF with Day 28 Response to Initial aGVHD Therapy

In CTN 0802 plasma samples, response to aGVHD therapy was associated with significant differences in circulating levels of EGF and VEGF-A at day 28 post-initial aGVHD therapy. EGF levels were 2-fold higher in patients with a complete response (CR, 20 pg/mL, versus 11 pg/mL for patients with partial response [PR] and 8 pg/mL for no response [NR], $P = .03$) (Figure 4). In addition, EGF levels significantly decreased from the onset of aGVHD to day 28 of therapy in patients who had NR ($P = .01$). Similarly for VEGF-A, patients with a CR had 2-fold higher levels at day 28 (CR, 163 pg/mL versus 79 pg/mL for PR and 86 pg/mL for NR, $P = .008$). In patients with CR to therapy, VEGF-A levels significantly increased from the onset of aGVHD to day 28 of therapy ($P = .006$). In contrast, in CTN 0302 serum samples, we observed no differences in EGF levels with response (CR, 46 pg/mL and PR, 62 pg/mL versus NR, 63 pg/mL, $P = .20$) nor VEGF-A levels (CR, 215 pg/mL and PR, 195 pg/mL versus NR, 166 pg/mL, $P = .80$). Importantly, the 0302 samples were serum (not plasma), which generally is associated with higher levels of these markers because of release from platelets [28,29].

Additionally, levels of Ang2 and sEng significantly decreased in patients experiencing a CR or PR at day 28 ($P < .001$ for each) across both cohorts. However, levels of Ang2 and sEng did not decrease ($P = .20$ and $P = .10$, respectively) in patients with NR at day 28 (Supplemental Figure 1).

Association of AF with 6-Month Survival

In CTN 0302 univariate survival analyses, elevated levels of sEng ($P = .04$) and Ang2 at aGVHD onset ($P = .02$) and at day 28 after initial aGVHD therapy ($P = .003$) were associated with lower 6-month survival. In CTN 0802, elevated FS ($P = .006$) and PlGF ($P = .006$) levels at day 28 after aGVHD therapy were associated with lower 6-month survival. These factors—Ang2, sEng, FS, and PlGF—were tested, along with

established important clinical variables for outcomes after allogeneic HCT, including patient age, aGVHD grade, and day 28 response to initial therapy [30], in a multivariate analysis of 6-month survival. In both trials, day 28 FS was an independent factor for 6-month survival (Table 2). The day 28 FS level of >2000 pg/mL showed the strongest association with poor survival (Figure 5). In CTN 0302, 80% of patients with a FS level below the cutoff were alive at 6 months versus 20% of patients with FS above the cutoff ($P = .001$). In CTN 0802, 83% of patients with a FS below the cutoff were alive at 6 months versus 58% with a FS above the cutoff ($P = .02$). In the controls without aGVHD, PlGF at day +100 was the only AF variable associated with survival, with poorer survival when the day 100 PlGF level was >40 pg/mL (relative risk, 49.5; 95% confidence interval, 2.0 to 1250; $P = .02$).

DISCUSSION

Without adequate levels of important trophic AF, host tissues may have compromised healing in the wake of severe aGVHD. Our study is the first to identify markedly low EGF levels at the onset of aGVHD. Low circulating EGF levels have been identified in IBD [9], which has similarities in presentation and perhaps pathogenesis with aGVHD [31]. Our results provide a rationale for supplementation of deficient factors to aid in repair as an adjunct to immunosuppression. The utility of AF replacement has been demonstrated with the use of EGF-containing enemas in left-sided ulcerative colitis [10]. The widespread tissue damage associated with aGVHD suggests that systemic administration of such factors may be needed. During steady state, the predominant luminal sources of EGF in the gastrointestinal tract are salivary and Brunner's glands, although Paneth cells, a known target of GVHD, have also been identified as producers of EGF [32,33]. We hypothesize that the low EGF levels we observed in GVHD are related to an alloimmune attack on EGF-producing cells, although increased EGF loss through

Table 2
Multivariate Analysis of Six-Month Survival

| Variable	BMT CTN 0302			BMT CTN 0802		
	HR	95% CI	P Value	HR	95% CI	P Value
Age > 40 yr	3.9	1.1-17.7	.03	3.3	1.1-14.6	.03
Grade			.17			.07
1	1.0	-		1.0	-	
2	2.0	.34-39.0		.7	.2-2.7	
3	5.1	.85-100.8		2.0	.5-8.7	
4	2.8×10^8	0-132.6		1.8×10^9	0-2.2	
Day 28 response			.01			.001
CR	1.0	-		1.0	-	
PR	.7	.2-2.6		1.7	.4-6.1	
NR	3.7	1.2-11.4		5.0	2.0-13.7	
Day 28 FS ≥ 2000 pg/mL	9.3	1.3-59.1	.002	2.8	1.04-6.9	.04
Onset Ang2 ≥ 8000 pg/mL	3.0	.6-11.7	.05	.3	.03-1.2	.09
Day 28 Ang2 ≥ 8000 pg/mL	.6	.05-5.1	.14	5.2	1.7-13.9	.006
Day 28 sEng ≥ 3000 pg/mL	.9	.04-8.5	.60	1.1	.5-2.6	.70
Day 28 PlGF ≥ 20 pg/mL	4.7	1.4-21.7	.005	1.8	.6-6.7	.30

HR indicates hazard ratio; CI, confidence interval.

protein-losing enteropathy or other mechanism is also possible.

Our initial pilot data were validated in 2 separate cohorts of patients from multicenter GVHD trials, lending external validity to the results. We also analyzed both serum and plasma in the different cohorts and were able to confirm that plasma is the preferred sample type for measuring EGF and VEGF-A. We note that only a few patients had severe grade III and IV aGVHD in the BMT CTN trials, and this may have compromised our ability to observe differences in these analyses with varying aGVHD severity. These validated findings associating AF, aGVHD response, and survival can be further hypothesis generating for future studies into the pathophysiology and therapy of aGVHD.

Levels of EGF and VEGF-A were significantly lower and more closely associated with response in CTN 0802, where plasma, as opposed to serum, was analyzed. Serum has higher levels of EGF and VEGF-A relative to plasma because of release from activated platelets [28,29]. Some of the differences we observed regarding AF and outcomes in the 2 trials may reflect the different sample sources, and further studies of these factors should ideally use plasma samples. Nonetheless, EGF serum levels were still significantly lower in aGVHD in CTN 0302 compared with the levels in recipient controls, which were 2 to 3 times the reported normal serum levels [22,23]. It is possible that increased EGF production beyond the normal basal state accompanies post-HCT recovery, but this requires further study.

In addition to the relative deficiency of circulating trophic growth factors for healing in aGVHD (EGF and VEGF-A), we also identified markers of endothelial damage after allogeneic HCT that were further dysregulated in aGVHD (FS and PlGF). Increased levels of circulating PlGF may reflect an underlying enhanced inflammatory state with higher levels of TNFα (rank correlation, .58; $P = .001$, unpublished), and increasing levels of FS may reflect greater endothelial damage and/or neovascularization. The results of this study contribute to the findings of endothelial-derived factors that are abnormal in aGVHD [34,35]. Interestingly, despite widespread inflammation and endothelial activation/injury, thrombosis is infrequent in severe aGVHD [36].

We observed that FS levels are significantly elevated after allogeneic HCT and are even higher after onset of aGVHD in both serum and plasma. The tissue source of excess FS in aGVHD is unknown. FS was first identified for its follicle-stimulating hormone inhibitory activity in ovarian follicular fluid [37], although it has more recently been shown to play a role in angiogenesis and function as a specific binding protein (and neutralizer) of activin-A, a ubiquitously expressed protein with proinflammatory and profibrotic properties that is elevated after allogeneic HCT [15,38,39]. However, FS is also a key regulator of tissue regeneration after injury [40]. Supplemental FS was recently shown to reduce mortality in rodent models of gram-negative sepsis [41] and IBD [42], and FS overexpression was associated with improved muscle healing after laceration [43]. We recently identified that circulating FS
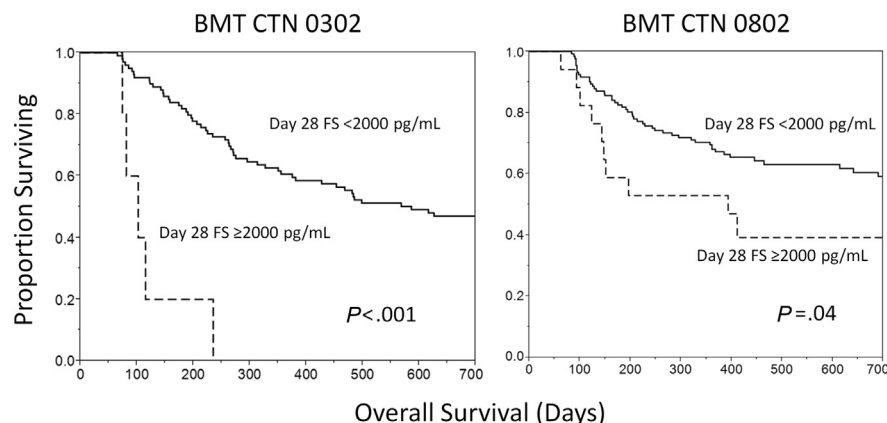
Figure 5. Two-year survival by follistatin (FS) level at day 28 of aGVHD.

is significantly higher in healthy pregnant women carrying a male fetus as opposed to those carrying a female fetus [44], suggesting that elevated FS may reflect adaptation to a greater immunologic challenge for the mother. Its association with poor outcomes in both GVHD trials suggest that marked elevations in FS may reflect endothelial damage [45,46], an established manifestation of steroid-refractory aGVHD [19], and/or neovascuarlization [43], which is associated with worsened aGVHD in rodents [5,6].

In summary, we observed that EGF and VEGF-A, factors involved in repair and regeneration, are low in aGVHD patients compared with levels in controls. EGF and VEGF-A are also associated with response to therapy, best recognized in plasma samples. AFs indicative of damage and inflammation, FS and PlGF, were elevated at the onset of aGVHD in both serum and plasma. Elevated FS at day 28 after initial aGVHD is associated with poor 6-month survival in both CTN 0302 and 0802. Ang2 and sEng decrease over time after effective aGVHD therapy. Finally, PlGF levels are disproportionately elevated in aGVHD after unrelated donor HCT but are also associated with overall survival in patients without aGVHD. Mechanistic studies are needed to clarify the role of these factors in post-HCT recovery, yet these data support the critical impact of the host healing capacity as well as endothelial damage for clinical response and survival after aGVHD.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2015.02.018.

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