High cutaneous amphiregulin expression predicts fatal acute graft-versus-host disease

Brittney Schultz | Daniel D. Miller | Todd DeFor | Bruce R. Blazar | Angela Panoskaltsis-Mortari | Brian C. Betts | Margaret L. MacMillan | Daniel J. Weisdorf | Shernan G. Holtan

1Department of Dermatology, University of Minnesota, Minneapolis, Minnesota, USA
2Hematology and Transplant, University of Minnesota, Minneapolis, Minnesota, USA
3Biostatistics and Informatics, University of Minnesota, Minneapolis, Minnesota, USA
4Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota, USA

Abstract

Background: Amphiregulin (AREG) is increased in circulation in acute graft-versus-host disease (aGVHD) and is associated with poor steroid response and lower survival. The expression of AREG in aGVHD target organs and its association with clinical outcomes are unknown.

Methods: We performed AREG immunohistochemical staining on skin specimens from 67 patients with aGVHD between the years 2010 and 2015. Two blinded reviewers assessed AREG expression and scored specimens with a semiquantitative scale ranging from 0 (absent) to 4 (most intense).

Results: Median AREG score of aGVHD cases was 3. Sixteen of 67 (23.9%) aGVHD cases had an AREG >3. High skin AREG expression (>3 vs. ≤3) was associated with increased overall clinical grade of aGVHD (52.9% vs. 33.4% clinical grade III-IV, \( p = 0.02 \)), reduced 3-year overall survival (OS; 13% vs. 61%, \( p < 0.01 \)), and increased 3-year non-relapse mortality (NRM; 56% vs. 20%, \( p = 0.05 \)).

Conclusion: High skin AREG immunohistochemical expression is associated with high clinical grade aGVHD, poor OS, and increased NRM.

Keywords

acute GVHD, allogeneic hematopoietic cell transplantation, amphiregulin, epidermal growth factor

1 | INTRODUCTION

Acute graft-versus-host disease (aGVHD) remains an important cause of morbidity and mortality after allogeneic hematopoietic cell transplant (HCT). The skin is the most commonly affected organ. Diagnosis, prognosis, and management of recurrent cutaneous aGVHD can be challenging. An immunohistochemical (IHC) stain that aids in determining the risk of fatal aGVHD would be of clinical utility.

Tissue repair responses are important determinants of clinical outcomes after aGVHD. One such tissue repair factor, amphiregulin (AREG), an epidermal growth factor (EGF) receptor ligand, is increased in both serum and plasma in aGVHD and is associated with poor steroid response and lower survival. EGF is often very low in plasma in steroid-refractory disease. AREG expression is increased in inflammatory disorders including psoriasis, and...
with important roles in tissue repair and immune response.\textsuperscript{8} Gastrointestinal (GI) AREG expression has recently been shown to be variable in GI aGVHD.\textsuperscript{9,10} The expression and role of AREG in human cutaneous aGVHD are unknown.

2 | MATERIALS AND METHODS

To define the association between AREG tissue expression and clinical outcomes, we analyzed serum samples and archived formalin-fixed, paraffin-embedded skin biopsy samples in post-HCT patients previously enrolled in the University of Minnesota clinical study “MT2009-22R: Monitoring of Immune Function and Minimal Residual Disease in Patients and Donors After Hematopoietic Cell Transplantation” (Principal Investigator: Michael Verneris, MD, USA). Patients were eligible for inclusion if they had a serum sample collected within 2 weeks prior to or following the onset of biopsy-confirmed cutaneous aGVHD ($n = 67$). Biopsy results from normal skin in non-HCT patients ($n = 10$) served as controls. Patients were selected by an expert reviewer (B. S.) following chart review of clinical history and dermatopathology reports. We deparaffinized and rehydrated unstained paraffin sections ($4 \mu m$) using standard methods. Subsequent steps were automated using an IHC staining platform (Nemesis, Biocare) using rabbit polyclonal anti-AREG (Biorbyt, San Francisco, CA, USA; 1:400) as the primary antibody. Slides were scored by two blinded expert reviewers (D. D. M. and B. S.) based on consensus score. The intensity of AREG nuclear expression in all cells (including keratinocytes, inflammatory cells) was determined with a semiquantitative score of 0 (absent) to 4 (most intense) with 0.5 gradations. Normal skin had no cases with AREG $>3$, and therefore we considered AREG above this level (scores 3.5 or 4.0) suggestive of pathology. Sera collected within 14 days of the skin biopsy of HCT cases were analyzed for concentrations of AREG (ELISA; R&D, Minneapolis, MN, USA) and EGF (multiplex bead array; Millipore, Billerica, MA, USA) according to the manufacturers’ instructions. Sera were not available for non-HCT controls.

We determined the association of AREG both by IHC and in the sera with clinical endpoints including overall survival (OS) and non-relapse mortality (NRM) as well as EGF in the sera. OS and NRM were calculated from the time of the biopsy (aGVHD diagnosis). Organ staging and clinical aGVHD grading was performed using modified Consensus criteria.\textsuperscript{1,11} Histopathologic grade of aGVHD was determined using modified Lerner criteria.\textsuperscript{12} Risk stratification was performed using the refined MN aGVHD Risk Score.\textsuperscript{1} Cox regression was used to examine the independent effect of AREG on OS. Fine and gray regression was used to examine the independent effect of AREG on NRM. Other factors considered in regression analyses were age, conditioning, donor type, Minnesota GVHD risk, comorbidity, and Karnofsky score. All reported $p$ values were two-sided. All analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). This study was approved by the University of Minnesota Institutional Review Board.

TABLE 1  Acute graft-versus-host disease patient demographics

|                           | All patients |
|---------------------------|--------------|
| N                         | 67           |
| Gender                    |              |
| Male                      | 39 (58%)     |
| Female                    | 28 (42%)     |
| Age                       |              |
| Median (range), (IQR)     | 50 (1–71), (33–59) |
| Donor type                |              |
| Matched sibling           | 20 (30%)     |
| MM sibling                | 2 (3%)       |
| Matched URD               | 10 (15%)     |
| sUCB                      | 5 (7%)       |
| dUCB                      | 30 (45%)     |
| Conditioning              |              |
| Myeloablative             | 37 (55%)     |
| Reduced intensity         | 30 (45%)     |
| GVHD prophylaxis          |              |
| CSA/MMF                   | 38 (57%)     |
| CNI/MTX                   | 17 (25%)     |
| Siro/MMF                  | 12 (18%)     |
| Diagnosis                 |              |
| Acute leukemia and myeloid malignancies | 55 (82%) |
| Lymphoid malignancies     | 8 (12%)      |
| Nonmalignant disorders    | 4 (6%)       |
| MN GVHD risk              |              |
| Standard risk             | 59 (88%)     |
| High risk                 | 8 (12%)      |
| Initial GVHD clinical grade |            |
| I                         | 25 (37%)     |
| II                        | 27 (40%)     |
| III                       | 14 (21%)     |
| IV                        | 1 (1%)       |
| Skin initial staging      |              |
| 1                         | 8 (12%)      |
| 2                         | 23 (34%)     |
| 3                         | 35 (52%)     |
| 4                         | 1 (1%)       |
| Median day following HCT when skin biopsy was obtained | 28.5 (interquartile range: Days 17–36) |

Abbreviations: AREG, amphiregulin; CNI, calcineurin inhibitor; CSA, cyclosporine; dUCB, double umbilical cord blood; GVHD, graft-versus-host disease; MM, mismatched, MMF, mycophenolate mofetil; MN, Minnesota; MTX, methotrexate; Siro, sirolimus; sUCB, single umbilical cord blood; URD, unrelated donor.

3 | RESULTS

Demographics of the 67 patients with histopathologic diagnosis of aGVHD are detailed in Table 1. The median day following HCT when skin biopsy was obtained was day 28.5 (interquartile range, Days 17–36). There were only four patients with aGVHD skin biopsies
beyond Day + 100 in this cohort; therefore this largely represents a classic aGVHD cohort. Figure 1 shows representative IHC stains. Median AREG score of aGVHD cases was 3. Sixteen of 67 (23.9%) aGVHD cases had an AREG >3. In all groups, expression of AREG was most prominent in keratinocytes and eccrine glands, although staining in lymphocytes was also observed.

High skin AREG expression (>3 vs. ≤3) was associated with increased overall aGVHD clinical grade (52.9% vs. 33.4% clinical grade III-IV, p = 0.02), reduced 3-year OS (13% vs. 61%, p < 0.01, Figure 2A), and increased 3-year NRM (56% vs. 20%, p = 0.05, Figure 2B). In multivariate analysis, patients with high skin AREG had a 3-fold increased risk of all-cause mortality (hazard ratio [HR] 3.0, 95% confidence interval [CI] 1.5–6.2, p < 0.01) and a 2.5-fold increased risk of NRM (HR 2.5, 95% CI 1.0–6.1, p = 0.05). High skin AREG did not correlate with Minnesota GVHD Risk (p = 0.38) or maximum skin aGVHD staging (p = 0.68). All patients except one had a serum AREG above the normal limit of 5 pg/ml (median 25.8 pg/ml, range 4.7–465.8 pg/ml). While there was no linear correlation between skin AREG staining and serum AREG in this cohort (Spearman’s rho –0.06, p = 0.7), high skin AREG was associated with low serum EGF (Spearman’s rho –0.34, p = 0.006), which is a negative prognostic marker of aGVHD.

4 | DISCUSSION

In this analysis, we showed that high skin AREG IHC expression is indicative of pathology and associated with high-grade clinical aGVHD, low serum EGF, poor OS, and increased NRM. This adds valuable information to our previous work associating high circulating AREG with poor clinical outcomes.4,5,13,14 This study also adds to our previous work detailing AREG expression in GI tissue in aGVHD. We previously found low AREG expression in GI mucosal epithelium and stoma in aGVHD biopsy samples compared to normal colon samples;
however, this study had largely non-severe GI aGVHD biopsy samples for analysis. We subsequently found high AREG expression by mRNA in the GI tract from patients with fatal aGVHD. Additionally, we found high circulating AREG protein and mRNA in peripheral blood mononuclear cells (PBMCs) in patients with fatal aGVHD. Thus, we hypothesize that although AREG protein expression in the colon normally acts to maintain the integrity of the epithelial barrier, it is a negative prognostic factor if it is highly expressed in the skin (pathologic) or found in circulation either by ELISA or PBMC gene expression studies (pathologic). The data overall suggest that high expression of AREG could indicate unresolved damage and/or is produced in response to danger signals, acting as an indicator of high-risk aGVHD when pathologically expressed outside the GI tract.

In addition to its potential role as a prognostic biomarker for aGVHD, there may be a therapeutic role of AREG in aGVHD. AREG is involved in enhanced tissue repair after injury. Indeed, protective effects of AREG in the setting of aGVHD have previously been proven in murine models, where the blockade of AREG led to increased intestinal aGVHD severity and mortality. AREG is also posited to have a role in immunity, as many cells in the innate and adaptive immune systems express AREG. AREG has been shown to enhance the action of Tregs, which may specifically have important consequences for aGVHD, as Tregs have been shown to prevent aGVHD. Furthermore, AREG was protective in the development of dermatitis in mice who were undergoing bone marrow transplantation.

Our study is strengthened by the blinded review and concomitant analysis of tissue and serum biomarkers. Although grading IHC staining remains subjective, it is readily determined to be pathologically elevated if increased relative to normal skin.

In summary, our results suggest that high skin AREG expression is a high-risk feature of aGVHD. High AREG expression in epidermal skin relative to normal skin should be further studied in a larger cohort as a prognostic aid in cutaneous aGVHD. Elevated tissue and serum AREG may be a reflection of the underlying tissue damage mediated by aGVHD, a counter-regulatory response to inflammation and/or reflection of danger signal sensing. Mechanistic studies are needed to understand the host response underlying elevated skin AREG and poor outcomes after aGVHD.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Brittney Schultz https://orcid.org/0000-0001-5080-892X

REFERENCES

1. MacMillan ML, Robin M, Harris AC, et al. A refined risk score for acute graft-versus-host disease that predicts response to initial therapy, survival, and transplant-related mortality. Biol Blood Marrow Transplant. 2015;21(4):761-767.
2. Zou Y, Barnett MJ, Rivers JK. Clinical significance of skin biopsies in the diagnosis and management of graft-vs-host disease in early posttransplantation bone marrow transplantation. Arch Dermatol. 2000;136(6):717-721.
3. Holtan SG, Verneris MR, Schultz KR, et al. 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. Biol Blood Marrow Transplant. 2015;21(6):1029-1036.
4. Holtan SG, Khera N, Levine JE, et al. Late acute graft-versus-host disease: a prospective analysis of clinical outcomes and circulating angiogenic factors. Blood. 2016;128(19):2350-2358.
5. Holtan SG, DeFor TE, Panoskaltsis-Mortari A, et al. Amphiregulin modifies the Minnesota Acute Graft-versus-Host Disease Risk Score: results from BMT CTN 0302/0802. Blood Adv. 2018;2(15):1882-1888.
6. Cook PW, Pittelkow MR, Keeble WW, Graves-Deal R, Coffey RJ, Shiple GD. Amphiregulin messenger RNA is elevated in psoriatic epidermis and gastrointestinal carcinomas. Cancer Res. 1992;52(11):3224-3227.
7. Piepkmn M. Overexpression of amphiregulin, a major autocrine growth factor for cultured human keratinocytes, in hyperproliferative skin diseases. Am J Dermatopathol. 1996;18(2):165-171.
8. Zajis DMW, Gause WC, Osborne LC, Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. Immunity. 2015;42(2):216-226.
9. Amin K, Yaqoob U, Schultz B, et al. Amphiregulin in intestinal acute graft-versus-host disease: a possible diagnostic and prognostic aid. Mod Pathol. 2019;32(4):560-567.
10. Holtan SG, Shabaneh A, Betts BC, et al. Stress responses, M2 macrophages, and a distinct microbial signature in fatal intestinal acute graft-versus-host disease. JCI Insight. 2019;5(17):e129762. doi:10.1172/jci.insight.129762.
11. Preziopkra D, Weisdo D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant. 1995;15(6):825-828.
12. Lerner KG, Kao GF, Storb R, Buckner CD, Cift RA, Thomas ED. Histopathology of graft-vs-host reaction (GvHR) in human recipients of marrow from HL-A-matched sibling donors. Transplant Proc. 1974;6(4):367-371.
13. Holtan SG, DeFor TE, Pidala J, et al. Amphiregulin improves stratification of the refined Minnesota acute graft-versus-host disease risk score: results from BMT CTN 0302/0802. Blood. 2017;130:72.
14. Holtan SG, Hoeschen AL, Cao Q, et al. Facilitating resolution of life-threatening acute GVHD with human chorionic gonadotropin and epidermal growth factor. Blood Adv. 2020;4(7):1284-1295.
15. Ramadan A, Griesenauer B, Adom D, et al. Specifically differentiated T cell subset promotes tumor immunity over fatal immunity. J Exp Med. 2017;214(12):3577-3596.
16. Zajis DM, van Loosdregt J, Gorlani A, et al. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. Immunity. 2013;38(2):275-284.
17. Di Ianni M, Falzetti F, Carotti A, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood. 2011;117(14):3921-3928.