THROMBOSIS AND HEMOSTASIS

Abnormalities in the alternative pathway of complement in children with hematopoietic stem cell transplant-associated thrombotic microangiopathy

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Key Points

- Genetic variations in the alternative pathway of complement may be associated with thrombotic microangiopathy in children receiving HSCT.
- These findings may guide the development of novel treatment interventions for this poorly understood transplant complication.

Hematopoietic stem cell transplant (HSCT)-associated thrombotic microangiopathy (TMA) is a complication that occurs in 25% to 35% of HSCT recipients and shares histomorphologic similarities with hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TPP). The hallmark of all thrombotic microangiopathies is vascular endothelial cell injury of various origins, resulting in microangiopathic hemolytic anemia, platelet consumption, fibrin deposition in the microcirculation, and tissue damage. Although significant advances have been made in understanding the pathogenesis of other thrombotic microangiopathies, post-HSCT TMA remains poorly understood. We report an analysis of the complement alternative pathway, which has recently been linked to the pathogenesis of both the Shiga toxin mediated and the atypical forms of HUS, with a focus on genetic variations in the complement Factor H (CFH) gene cluster and CFH autoantibodies in six children with post-HSCT TMA. We identified a high prevalence of deletions in CFH-related genes 3 and 1 (delCFHR3-CFHR1) and CFH autoantibodies in these patients with HSCT-TMA. Conversely, CFH autoantibodies were not detected in 18 children undergoing HSCT who did not develop TMA. Our observations suggest that complement alternative pathway dysregulation may be involved in the pathogenesis of post-HSCT TMA. These findings shed light on a novel mechanism of endothelial injury in transplant-TMA and may therefore guide the development of targeted treatment interventions. (Blood. 2013;122(12): 2003-2007)

Introduction

Hematopoietic stem cell transplant (HSCT)-associated thrombotic microangiopathy (TMA) shares histomorphologic similarities with other small vessel diseases including diarrhea positive hemolytic uremic syndrome (HUS), atypical HUS (aHUS), thrombotic thrombocytopenic purpura (TPP), and preeclampsia/HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets). These disorders occur when endothelial injury leads to microangiopathic hemolytic anemia, platelet consumption, fibrin deposition in the microcirculation, and, ultimately, end-organ injury.¹,²

Advances in understanding the pathogenesis of other microangiopathies, such as the role of complement mutations in aHUS and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) deficiency in TTP, has improved outcomes through early diagnosis and the use of targeted therapies.³-⁵ In contrast, although TMA was recognized as a complication of HSCT in the early 1980s and may occur in 25% to 35% of stem cell transplant recipients, we do not fully understand the exact mechanisms of vascular endothelial injury in HSCT-associated TMA.⁶-⁸ Risk factors for HSCT-associated TMA include chemotherapy, radiation, graft versus host disease, calcineurin inhibitors, and viral infections.¹,⁹ The kidney is the most commonly injured organ, but unrecognized and untreated HSCT-associated TMA can evolve into a lethal multisystem disease.¹⁰ In its most severe form mortality rates are very high (~90%), especially when dialysis is required. Milder cases may increase the risk of later chronic kidney disease.¹¹,¹²

Dysregulation of the complement alternative pathway has been implicated in the kidney injury found in patients with Shiga toxin mediated and aHUS, membranoproliferative glomerulonephritis (MPGN), and preeclampsia/HELLP syndrome.¹³,¹⁴ Inappropriate complement activation or insufficient inhibition can result in vascular endothelial injury and thrombotic microangiopathy. Deletions in the complement Factor H (CFH)-related genes 3 and 1 (delCFHR3-CFHR1) have been associated with autoantibodies


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against CFH in a subgroup of patients with aHUS called DEAP HUS (Deficiency of CFHR plasma protein and Autoantibody Positive) who respond to antibody depleting or complement blocking therapy.\textsuperscript{5,16} The role of complement in TMA occurring after stem cell transplantation has not been defined. We present our clinical observations suggesting the complement alternative pathway is involved in the pathogenesis of HSCT-associated TMA.

**Methods**

**Patient population**

Six consecutive patients who developed TMA after HSCT at Cincinnati Children’s Hospital Medical Center (CCHMC) in 2009 were tested to determine the potential role of the complement alternative pathway in HSCT-associated TMA. TMA was diagnosed using current diagnostic criteria including elevated lactate dehydrogenase (LDH) above normal for age, haptoglobin below the lower limit of normal, evidence of schistocytes on peripheral blood smear, anemia, thrombocytopenia, a negative Coombs test, and acute kidney injury (AKI) defined as a doubling of the serum creatinine relative to each subject’s pre-HSCT baseline.\textsuperscript{17,18} Informed consent for clinical genetic testing was obtained according to the requirements of the Molecular Otolaryngology & Renal Research Laboratories (MORL) at the University of Iowa. Research informed consent was obtained from patients for CFHR protein testing on stored serum samples, and the CCHMC Institutional Review Board approved data review and analysis. Study subjects’ pre-HSCT plasma samples and control subjects’ plasma samples were obtained from the CCHMC Bone Marrow Transplant repository. All subjects in the repositories had previously consented to “future research studies.” The control group included 10 consecutive allogeneic HSCT recipients without any evidence of TMA or AKI during the first 100 days post-HSCT. 5 consecutive allogeneic HSCT recipients with AKI of non-TMA origin documented as doubling of serum creatinine from pre-HSCT baseline, but without any laboratory evidence of TMA, and 3 patients after autologous stem cell transplantation without any evidence of TMA or AKI. This study was conducted in accordance with the Declaration of Helsinki.

**Complement gene testing**

Recipient DNA was extracted from blood samples collected before HSCT. Direct gene sequencing analysis was used to identify single nucleotide variations, small deletions, and small insertions in the protein coding regions including intron/exon boundaries of CFH, Complement Factor I (CFI), membrane cofactor protein (MCP), Complement Factor B (CFB), and CFH-related-5 (CFHR5) genes. Multiplex Ligation-Dependent Probe Amplification (MLPA) testing procedure was used to examine CFH-CFHR5 in order to identify two common, large deletions in the regulator of complement activation (RCA) locus, delCFHR3-CFHR1 and delCFHR1-CFHR4, in both recipient and donor pre-HSCT samples. All complement gene testing was performed at the MORL laboratory according to their established laboratory techniques. Results were reported as the presence or absence of alleles. The presence of both alleles was considered a normal test. The absence of one allele was reported as a heterozygous gene deletion, and the absence of both alleles was reported as a homozygous gene deletion. ADAMTS13 activity was tested in all patients to rule out TTP by fluorescence resonance energy transfer (FRET) at CCHMC clinical laboratory.

**Autoantibodies to CFH**

In the six study subjects who developed TMA, CFH autoantibody was tested on plasma collected before HSCT and after diagnosis of TMA. CFH autoantibody testing for the control group without a diagnosis of TMA was performed on plasma specimens collected at 100 days after HSCT, because the highest risk time to develop TMA is during the first 100 days after transplantation. The presence of antibodies to the full CFH protein was tested using an enzyme-linked immunosorbent assay (ELISA) at the MORL laboratory for study subjects and CCHMC laboratory for control group according to their established techniques. Results were reported as a “present” or “absent” CFH autoantibody.

**Analysis of circulating CFHR1**

In the six study subjects with TMA, serum was tested for the presence of the CFHR1 protein on samples collected before HSCT. Testing was performed by immunoblotting (western blot) as previously described at the Division of Nephrology, The Hospital for Sick Children, Toronto, ON, Canada.\textsuperscript{14} Results were reported as “present” or “absent” CFHR1.

**Results**

The clinical characteristics of the six consecutive HSCT patients with diagnosis of TMA are summarized in Table 1. Most of the patients underwent HSCT for a malignant disorder (5 out of 6, 83%).
patients received an allogeneic HSCT from an unrelated donor, and the remaining three received an autologous HSCT. TMA was diagnosed clinically a median of 32.5 days post-HSCT (range 9–111 days). The diagnosis of TMA was also confirmed by kidney biopsy in two subjects (patients 1 and 4).

Table 1 also lists the complications associated with TMA, therapies received, and clinical outcome. All patients developed AKI and had severe hypertension requiring 4 to 9 antihypertensive medications to control their blood pressure. Three of the six patients (patients #1, #4, and #6) required hemodialysis. Regarding potential triggers for TMA, three subjects (patients 1, 4, and 6) had CMV viremia, parainfluenza 3, and adenoviremia, respectively, before their diagnosis of TMA, but no other documented infections. None of the three allogeneic recipients had evidence of graft versus host disease.

Calcineurin inhibitor prophylaxis was stopped in the three allogeneic recipients after TMA was diagnosed. Weekly rituximab (375 mg/m²/dose) infusions were empirically initiated in all subjects and 2 to 10 doses were administered. Four patients with continued clinical evidence of thrombotic microangiopathy despite rituximab administration received daily therapeutic plasma exchange (TPE) according to the institutional practices, as previously described. Four of the six patients (66%) had a positive response to rituximab and/or TPE, whereas the remaining two progressed to end-stage kidney disease.

The results of the analysis of the complement alternative pathway are shown in Table 2. Five of the 6 transplant recipients (83%) had heterozygous CFHR3-CFHR1 gene deletions detected by MLPA. On further testing, one of these five patients was shown to have a heterozygous deletion spanning from CFHR1-CFHR4. Three autologous transplant recipients with TMA were previously reported, as part of a larger neuroblastoma cohort, as having no detectable complement gene abnormalities, because they had no CFI, CFH, MCP, CFB, or CFHR5 genes by direct gene sequencing, but CFH-CFHR5 testing by MLPA was not available at that time, and these results were reported to us by MORL laboratory after initial publication. One of the bone marrow donors (1 out of 3, 33%) also had a heterozygous deletion in the CFHR3-CFHR1 gene detected by MLPA. All three allogeneic transplant recipients had detectable CFH autoantibodies. Two of these patients with CFH autoantibodies had an associated heterozygous CFHR3-CFHR1 deletion, whereas the remaining allogeneic transplant recipient did not have any detectable complement gene abnormalities that were tested. Patient #6 was previously described as a clinical case report showing complete resolution of hyperacute TMA with multiorgan involvement that completely responded to prompt therapy with TPE and rituximab. This patient is doing clinically well >4 years from TMA diagnosis.

Patients #4 and #5 are newly identified cases with CFH autoantibody in our cohort of consecutive patients tested for complement system abnormalities resulting from TMA. None of the patients in our cohort had identifiable mutations in CFI, CFH, MCP, CFB, or CFHR5 genes by direct gene sequencing. By western blot, all patients had detectable CFHR1 protein. There were no available tests to examine CFHR1 function. All subjects had normal or elevated serum levels of C3 and C4 at TMA diagnosis. ADAMTS13 activity was normal to slightly decreased, ranging from 59% to 96%, ruling out a diagnosis of TTP (normal range >67% with <10% being diagnostic for TTP). Four of six patients (#2, #3, #4, #5) had pre-HSCT plasma sample available in the BMT repository that had no detectable CFH autoantibody before starting transplant chemotherapy. None of the control group plasma samples were positive for CFH autoantibody at 100 days after HSCT. Overall, 3 out of 6 (50%) of the patients with TMA had detectable CFH autoantibodies, compared with 0 out of 18 (0%) of the controls (P = .01).

### Discussion

We examined the complement alternative pathway in 6 children developing TMA after either autologous or allogeneic HSCT. We identified a high prevalence (83%) of heterozygous CFHR3-CFHR1 deletions in HSCT recipients with TMA, whereas the same gene variations in donors (33%) occurred at a frequency similar to that reported in the general population. Additionally, all three allogeneic HSCT recipients with TMA had detectable autoantibodies to CFH, two of whom had associated heterozygous CFHR3-CFHR1 deletions. We speculate that CFH autoantibodies detected after HSCT are pathogenic and can possibly trigger TMA, because 2 of these 3 allogeneic HSCT recipients, for whom pre-HSCT plasma samples were available, were shown to have CFH autoantibody at TMA diagnosis but not before transplantation. Also, none of the HSCT recipients without TMA (control group) had any detectable CFH autoantibodies at 100 days after transplantation, suggesting that CFH autoantibodies may play role in the development of TMA after HSCT.

The exact pathogenesis of TMA after HSCT remains incompletely understood, limiting identification of the patients at highest risk for this transplant complication and also the appropriate selection of targeted therapies. Although TMA after HSCT may be triggered by several factors, our preliminary observations suggest that TMA may be associated with complement dysregulation at least in some HSCT recipients, as evidenced by detectable CFH autoantibodies and a positive response to antibody depleting therapy (rituximab, TPE) in our patients.

Autoimmunity is a well-recognized complication after allogeneic stem cell transplantation, and CFH autoantibodies could potentially be produced by host plasma cells as a response to recipient/donor CFH genotype differences, as CFH autoantibodies were only observed in allogeneic but not in autologous HSCT recipients in our cohort.
Inhibitory autoantibodies to CFH, either alone or in combination with complement gene mutations, have been reported in patients with aHUS. In aHUS, homozygous mutations in the CFHR3-CFHR1 genes have been associated with CFH autoantibody formation, an entity termed DEAP-HUS (Deficiency of CFHR plasma protein and Autoantibody Positive). Homozygous CFHR3-CFHR1 deletions are reported in 2% to 5% of the normal healthy white population, are observed in 11% to 15% of patients with aHUS, and occur in >90% of patients with DEAP-HUS. By contrast, heterozygous CFHR3-CFHR1 deletions are identified in 20% to 24% of whites and up to 35% of African Americans.

Patients with aHUS lacking CFHR1, but not those lacking CFHR3, present with CFH antibodies, suggesting that the generation of these antibodies is associated with CFHR1 deficiency. Although CFH autoantibodies are reported in approximately 10% of patients with aHUS, they are not specific for aHUS and have been identified in 9% to 16% of patients with rheumatoid arthritis, 7% of patients with systemic lupus erythematosus, 9% of patients with a history of thrombosis and antiphospholipid antibodies, and 4% of adult volunteers in a large European study. The same study reported that in patients with clinical disease, CFH autoantibody titers varied among individual patients throughout time and were usually detected during periods of more severe disease.

CFH autoantibodies isolated from patients with aHUS bind to the C-terminus of CFH. As in aHUS-related CFH mutations, which also affect the C-terminal short consensus repeats (SCRs) 19-10 of CFH, CFH autoantibodies prevent CFH from attaching to the surfaces, thus resulting in a lack of normal complement control on the endothelium. In contrast, epitope mapping experiments in patients with rheumatic disease suggest that CFH autoantibodies bind to several epitopes scattered throughout the CFH protein, possibly explaining the more generalized inflammation observed in patients with rheumatic conditions relative to aHUS in which the kidney is preferentially injured.

Our observation that heterozygous CFHR3-CFHR1 deletions were identified in a high percentage of HSCT patients with TMA and were associated with detectable CFH autoantibodies in allogeneic HSCT recipients warrants further investigation. We speculate that heterozygous deletions in CFHR3-CFHR1 genes, even though common in the general population, may influence recipients’ susceptibility to direct endothelial injury from high-dose chemotherapy or viral infections after HSCT. It would therefore be of clinical interest to determine whether recipient, donor, or both genotypes determine the susceptibility to TMA after HSCT, potentially supporting baseline genetic testing of the complement alternative pathway.

Because CFH autoantibodies were checked only once after the diagnosis of TMA in our patients, we were not able to correlate the presence of CFH autoantibodies with disease severity or to determine if CFH autoantibodies were simply missed in autologous HSCT recipients. Because autoimmunity is a well-documented phenomenon after allogeneic HSCT, it remains possible that the presence of autoantibodies to CFH may reflect immune dysregulation after HSCT independent of the genetic abnormalities in the complement system described in other types of thrombotic microangiopathies.

Clinically, two allelic and two autologous HSCT recipients (4/6, 67%) responded favorably to empiric therapy depleting pathogenic antibodies (rituximab, TPE). Even though CFHR1 was detected in all patients by western blot, we were unable to determine whether this protein is fully functional in patients with heterozygous CFHR3-CFHR1 deletions. Since the CFHR1 protein is a presumed regulator of the C5 convertase within the complement alternative pathway, it would be important later to examine its function in individuals with heterozygous CFHR3-CFHR1 deletion and to determine if TPE offers therapeutic benefit by replacing defective protein in these patients. Recent studies showed that exogenous CFHR1 provided during plasma exchange therapy for thrombotic microangiopathy may neutralize anti-factor H autoantibodies and help in the treatment of autoimmune aHUS. Such knowledge would lead to a more rational use of rituximab and TPE treatments in HSCT patients with TMA for which we currently have no clear understanding of their therapeutic mechanisms.

Along these lines, novel therapies such as the terminal complement inhibitor eculizumab may offer another treatment option for post-HSCT TMA. Eculizumab, which binds to C5 and prevents generation of the membrane attack complex, has shown benefit in patients with antibody-mediated kidney transplant rejection, aHUS, and preeclampsia/HELLP syndrome and was recently reported to abrogate HSCT-associated TMA in an adult patient without a documented complement genetic abnormality. Targeted therapy aimed at minimizing vascular endothelial damage in patients with TMA may preserve kidney function and improve outcomes after HSCT. This is especially relevant for children who have a lifetime of increased risk of developing chronic kidney disease after HSCT. Future research is needed to examine further the role of complement in the pathogenesis of TMA.

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Authorship
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