CLINICAL SCIENCE

Does minor histocompatibility antigen HA-1 disparity affect the occurrence of graft-versus-host disease in tunisian recipients of hematopoietic stem cells?

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INTRODUCTION: Minor histocompatibility antigen HA-1 (MiHAg-HA-1) disparity between a patient and his or her human leukocyte antigen (HLA) genoidentical donor has been widely associated with an increased risk of graft-versus-host disease following allogeneic hematopoietic stem cell transplantation.

OBJECTIVE: To examine the effect of HA-1 disparity on the incidence of both acute and chronic graft-versus-host disease in Tunisian recipients of hematopoietic stem cells.

METHODS: A total of 60 patients and their 60 respective sibling hematopoietic stem cell donors were enrolled in this study. All patients prophylactically received cyclosporine A and/or methotrexate for graft-versus-host disease. An HA-1 genotyping assay was performed with the SSP-PCR method, and HLA-A*0201- and/or HLA-A*0206-positive samples were identified using the Luminex HLA typing method.

RESULTS: The Luminex HLA typing assay showed that 54 patients were positive for either the HLA-A*0201 or HLA-A*0206 alleles. Among these cases, six pairs were mismatched for MiHAg-HA-1. Both acute and chronic graft-versus-host disease occurred in four mismatched patients (Fisher's p-values were 0.044 and 0.170, respectively). A univariate logistic regression model analysis showed that only acute graft-versus-host disease may be affected by recipient MiHAg-HA-1 disparity (p: 0.041, OR: 6.727), while chronic graft-versus-host disease correlates with both age and recipient/donor sex mismatch (p: 0.014, OR: 8.556 and p: 0.033, OR: 8.664, respectively).

CONCLUSION: Our findings support previously reported data suggesting a significant association between HA-1 disparity and the risk of acute graft-versus-host disease following hematopoietic stem cell transplantation.

KEYWORDS: Hematopoietic stem cell transplantation; Graft-versus-host disease; Minor histocompatibility antigens; HA-1; HLA-A; Tunisian population.

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INTRODUCTION

Graft-*versus*-host disease (GVHD) is the major cause of mortality and morbidity following an allogeneic hematopoietic stem cell transplantation (HSCT).[1–4]

This complication has been widely linked to immunogenetic causes, such as a disparity between the recipient and his or her human leukocyte antigen (HLA) genoidentical donor for certain non-HLA antigens, called minor histocompatibility antigens (MiHAgs).[2,4–6] These antigens are endogenously synthesized polymorphic products that can be recognized by alloreactive T cells only in the context of major histocompatibility complex molecules (HLA).[6–9] The importance of MiHAgs, encoded by sex-linked or autosomal loci, in the occurrence of GVHD has been demonstrated previously.[2–4,6]

Disparity in a limited number of immunodominant MiHAgs may be sufficient to cause acute GVHD, but the available data do not permit definitive conclusions.[5,10–16] Donor/recipient disparity for HA-1, the most extensively studied MiHAg and whose expression is limited to hematopoietic cells, was initially correlated with acute GVHD in studies involving small numbers of patients.[5,12–14] In analyses of larger cohorts, however, this disparity was not significantly associated with the development of acute GVHD.[17–20] Thus, additional studies in other populations

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of the same area of research are needed to confirm the previous result and define the effect of HA-1 disparity on GVHD occurrence following HSCT.

The minor histocompatibility antigen HA-1 was initially identified by a cytotoxic T cells clone that was recovered from a marrow transplant recipient with GVHD.[21] This antigen is a nonapeptide that is part of a protein encoded by the *HMHA1* gene that maps to chromosome 19p13.3.[22] This gene has two allelic versions differing at positions 500 (rs3764653) and 504 (rs1801284) of the cDNA sequence, resulting in a single amino acid polymorphism.[23] The HMHA1^{H} allele encodes histidine (HA-1^{H}) at position 3 of the peptide (VLHDDLLEA), whereas the HMHA1^{R} allele encodes arginine (HA-1^{R}) at the same position. The HLA-A*0201 and HLA-A*0206 molecules have high affinities for the HA-1^{H} peptide, and the generated antigen/MHC complex is recognized by HA-1-specific cytotoxic T cells.[23,24] Unlike this peptide, the HA-1^{R} peptide does not generate a detectable immune response.[23] HA-1-positive individuals can be either homozygous or heterozygous carriers of the HA-1^{H} peptide. Thus, an HA-1 mismatch is defined by the presence of the HA-1^{H} allele in the host and not the donor.

Tunisia is a small North African country characterized by a mixed origin and a high rate of consanguinity.[25,26] Recently, we performed a population genetic study, focusing on *HMHA-1* gene polymorphisms in Tunisians.[27] In this study, we found a noticeable probability that a Tunisian recipient of HSCs may be positive for HA-1/HLA-A*0201. Consequently, we examined the effect of HA-1 mismatch on the occurrence of GVHD. To achieve this purpose, a cohort of HSC recipients and their HLA-identical siblings was selected to conduct this retrospective study.

MATERIALS AND METHODS

To participate in this study, informed consent was obtained from all participants and/or their families according to a protocol approved by the Ethical Committee for Scientific and Medical Research of the National Blood Transfusion Center of Tunis (Tunisia).

Patient selection

Because HA-1 antigen presentation is restricted to the HLA-A*0201 and HLA-A*0206 molecules, we selected only HLA-A2-positive samples to perform this study. The cohort comprised 120 individuals: 60 patients and their 60 respective sibling hematopoietic stem cell donors. All subjects were Tunisian. The patients underwent HLA-matched HSCT in the National Bone Marrow Transplantation Centre of Tunis between 2000 and 2009. All patients prophylactically received cyclosporine A and/or methotrexate for GVHD and had grades 0–I or II–IV acute GVHD. See Table 1 for other clinical characteristics.

DNA samples

EDTA-treated blood samples were collected from all individuals prior to HSCT. DNA was purified from peripheral blood leukocytes using a DNA Blood Mini Kit (*Qiagen*, *Leiden*, *the Netherlands*) according to the manufacturer's instructions.

GVHD grading

The diagnosis and grading of acute and chronic GVHD were defined according to Seattle Transplant Team criteria.[28]

Table 1 - Clinical characteristics of the patient/donor pairs.

Patient age (years)	Median: 24.00 Range: 4–49	**Patient sex**	(MF) 2826
Sex combination (patient/donor)	M/M: 13 M/F: 16 F/F: 11 F/M: 14	**Patient age (< or >16 years)**	<16: 17 >16: 34 Missing data: 3
Diagnoses	02: CML 21: AML 11: ALL 06: FA 01: MDS 10: AA	**Conditioning**	24: BU + CY 11: TBI + CY 11: ATGAM + CY 03: FL + CY 04: TBI + VP16 01: BU + VP16 + CY
	03: MM	**Prophylactic GVHD treatment**	44: CSA + MTX 10: CSA

Abbreviations: M, male; F, female; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; FA, Fanconi's anemia; MDS, myelodysplastic syndrome; AA, aplastic anemia. MM, multiple myeloma; BU, busulphan; CY, cyclophosphamide; TBI, total body irradiation; FL, fludarabine; CSA, cyclosporin A; MTX, methotrexate.

HA-1 genotyping

HA-1 genotyping was performed using a previously described allele-specific PCR method.[22] This SSP-PCR method uses two sets of primers: set 1 has a forward common primer and two allele-specific reverse primers, and set 2 has a reverse common primer and two allele-specific forward primers. The allele-specific primers target the polymorphic region that encodes the HA-1^{H} or HA-1^{R} allele. All primers were synthesized as oligonucleotides (*Invitrogen Corporation*, *CA*, *USA*).

PCR amplification was carried out in a final volume of 10 µL containing 200 µM of each dNTP, 0.2 µM of each primer (which detects a specific allele), 0.2 µM of control primers (which detect a sequence from the HGH allele), and 1.5 mM $MgCl_2$ in 2 µL of 5× Green Buffer (*Promega Corporation*, *USA*). Each PCR was performed using 150 ng of genomic DNA and 0.4 units of Taq polymerase (*GoTaq^{TM} DNA Polymerase*, *Promega Corporation*, *USA*).

The amplification protocol was 94℃ for 5 min followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 61℃ for 40 s, and elongation at 72℃ for 30 s; the final elongation step was 72℃ for 5 min (*Gene Amp PCR System 9600*, *Perkin Elmer*, *CT*, *USA*). The PCR products were visualized on agarose gels (1.5%) with UV illumination and photodocumented (*UViTech^{TM}*, *Germany*).

HLA-A*0201/HLA-A*0206 allele typing

Because HA-1 antigen presentation is restricted to HLA-A*0201 and HLA-A*0206 molecules, only carriers of these alleles were selected for the statistical analysis. All patients and donors were previously typed as HLA-A2-positive using standard clinical laboratory techniques with serological or molecular testing methods (*One Lambda*, *USA and Innogenetics*, *Belgium*). HLA-A*0201 and HLA-A*0206 allele typing was initially performed with Luminex^{TM} technology (*One Lambda Corporation*, *USA*) and was confirmed in some cases with the SSP-PCR method (*One Lambda Corporation*, *USA*).

Statistical analysis

The association between HA-1 mismatch and the incidence of either acute or chronic GVHD was evaluated using Fisher's exact test. The odds ratios for univariate and

multivariate logistic regression models were used to analyze the correlations between risk factors and the probabilities of acute and chronic GVHD. All statistical analyses were performed with SPSS software (v. 17.0).

RESULTS

Among 60 HLA-A2-positive patient/donor pairs, the Luminex™ molecular genotyping assay showed that 53 cases were positive for the HLA-A*0201 allele, and 1 pair carried the HLA-A*0206 variant. The remaining six cases expressed the HLA-A*0205 allele and were excluded from the statistical analysis. Thus, 54 pairs were carriers of the specific HLA molecules needed for HA-1 peptide presentation. When typed for this MiHAg, only six pairs were mismatched (11.11%) *vs.* forty-eight identical pairs (88.89%). Acute GVHD of grades II–IV occurred in only four HA-1 mismatched recipients *vs.* eleven matched pairs (Table 2). Like the majority of cases, these four patients had received CSA and MTX as prophylaxis for GVHD. When treated in a univariate logistic regression model, HA-1 disparity appeared to be correlated with an increased risk of acute GVHD (*p*: 0.041, OR: 6.727) but showed only a trend toward significance in the multivariate logistic regression analysis. Furthermore, acute GVHD was not influenced by factors such as sex mismatch, conditioning and patient age. All results are summarized in Tables 2 and 3.

With regard to chronic GVHD, this outcome occurred in 13 HA-1-matched pairs *vs.* only 4 mismatched pairs. When we compared its incidence between HA-1-matched and mismatched recipients, we found no significant difference. This finding was determined from a statistical analysis using Fisher's exact test and verified with univariate and multivariate logistic regression models (Tables 2 and 3). Interestingly, chronic GVHD may be significantly promoted by both donor/recipient sex mismatch and patient age (adulthood). A total of 82.4% of patients who developed chronic GVHD were sex mismatched with their siblings, and 94.11% of patients who developed this outcome were adults. None of the other risk factors, including conditioning and malignancy, were significant in the paradigm of chronic GVHD incidence.

DISCUSSION

GVHD is one of the major complications of allogeneic HSCT, even in an HLA genoidentity context.[1–4] Disparity between a recipient and his or her HLA-identical sibling for certain minor histocompatibility antigens may be sufficient to cause GVHD following HSCT.[2,4–6] Based on these data, we investigated the effect of recipient MiHAg-HA-1 disparity on GVHD occurrence in a Tunisian series of HSC allografts. After screening an HLA-A*0201/HLA-A*0206-positive cohort, we performed HA-1 typing for each recipient/donor pair. The correlation analysis showed that acute GVHD may be affected by HA-1 mismatch because Fisher's *p*-value for this relationship was 0.044. This result was confirmed by the univariate logistic regression analysis, but in the multivariate regression model, we noticed only a trend toward an increased probability of Acute GVHD of grades II–IV. The same multivariate analysis showed no significant association between risk factors such as patient age, conditioning or sex mismatch and the incidence of acute GVHD. As expected, our data support previously reported findings correlating acute GVHD occurrence with recipient HA-1 disparity,[5,12–14] but a definitive conclusion cannot be deduced. This fact may be explained by the prophylactic protocol received by patients enrolled in each study. Thus, we believe that the actual effect of HA-1 disparity cannot be evaluated in subjects who receive CSA and MTX to prevent GVHD occurrence. Likewise, differences in the methods used for sample selection or GVHD grading could affect the level of significance. In our opinion, to assess this relationship more accurately, we need to investigate the effect of HA-1 disparity in a universal context, particularly in patients who have not received GVHD prophylaxis. We believe that this procedure may be feasible if it is performed first in murine models and then in human patients.

Our study showed that chronic GVHD may be affected by both donor/recipient sex mismatch and adulthood but not by HA-1 disparity. Statistically, 82.4% of patients who developed chronic GVHD were sex mismatched with their HLA-identical siblings (recipient sex-ratio: 1.33). Furthermore, patient age appeared to be a critical player in chronic GVHD onset because 94.11% of those patients who developed chronic GVHD were adults. Both sex mismatch and adulthood have been described as favorable conditions for the occurrence of these effects, especially through T cells

Table 2 - Effect of HA-1 mismatch on the occurrence of acute and chronic GVHD in Tunisian HSC recipients.

	Acute GVHD status		Fisher's *p*-value
	Grades 0–I	*Grades II–IV*	*p* = 0.044
HA-1 mismatched pairs (*n*)	2 (*33.3%*)	4 (*66.7%*)	
HA-1 matched pairs (*n*)	37 (*77.1%*)	11 (*22.9%*)	
	Chronic GVHD status		*p* = 0.170
	No	Yes	
HA-1 mismatched pairs (*n*)	2 (*33.3%*)	4 (*66.7%*)	
HA-1 matched pairs (*n*)	28 (*68.3%*)	13 (*31.7%*)	

Table 3 - Multivariate logistic regression model of risk factors for grades II–IV acute and chronic GVHD.

	Acute GVHD			
			95% CI	
	p	**OR**	**Lower**	**Upper**
HA-1 mismatch	0.055	6.323	0.964	41.464
Adult status: < or >16 years of age	0.671	1.355	0.333	5.510
Donor/recipient sex mismatch	0.654	1.350	0.364	5.013
Disease: malignant *vs.* nonmalignant¥	0.560	0.632	0.135	2.957
Conditioning with TBI	0.630	1.470	0.307	7.032
	Chronic GVHD			
			95% CI	
	p	**OR**	**Lower**	**Upper**
HA-1 mismatch	0.091	8.765	0.709	18.391
Adult status: < or >16 years of age	**0.014**	**8.556**	1.820	29.193
Donor/recipient sex mismatch	**0.033**	**8.664**	1.192	22.974
Disease: malignant *vs.* nonmalignant	0.060	11.257	0.900	40.818
Conditioning with TBI	0.836	0.819	0.123	5.456

Modeled according the default method and Hosmer-Lemeshow goodness-of-fit
¥malignant: CML, AML, ALL, MDS, MM; nonmalignant: FA, AA (see abbreviations in Table 1)

alloreactivity against Y-encoded antigens when the recipient/donor pairs have different genders.[5,29–31] Thus, our findings are in agreement with those findings reported by Gallardo et al.; in their large pool of patients, they found no association between chronic GVHD and HA-1 disparity.[13]

On the other hand, chronic GVHD may be affected by acute GVHD status. This outcome occurred in 53% of patients who developed acute GVHD (*data not shown*). This fact may be explained by the presence of T cells that are primed by chronic antigen stimulation due to the presence of ubiquitous MiHAgs.[3] Our findings support previous data qualifying acute GVHD as the main risk factor for the development of chronic GVHD,[32] but future studies are needed to investigate the role of regulatory T cells, B cells and other immune cells. In this regard, recent data have suggested a critical role for humoral immunity in the chronic GVHD paradigm because there is a strong correlation between this outcome and both increased numbers of B cells with altered Toll-like Receptor 9 (*TLR9*) responses and the presence of antibodies against some minor histocompatibility antigens.[30,32–33]

In conclusion, this study showed that acute GVHD but not chronic GVHD may be affected by HA-1 disparity in Tunisian HSC recipients. Consequently, we believe that HA-1 typing may be useful in cases where there is more than one HSC donor, which occurs frequently in Tunisian families (*data not shown*). Overall, we believe that our results are significant and can encourage further study in this area of cell therapy in Tunisia because this study is the first to report the role of immunogenetic markers in the occurrence of GVHD. It will be important, however, to expand the patient cohort and investigate the effects of HA-1 disparity on other endpoints, including leukemia relapse, disease-free survival and overall survival.

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ELECTRONIC DATABASE INFORMATION

Accession numbers and URLs for data in this article are as follows:

- GenBank: http://www.ncbi.nlm.nih.gov/GenBank/ (HA-1 *Gene ID*: 23526)
- EnsemblGene: http://www.ensembl.org/ (HA-1 *Ensembl Gene ID*: ENSG00000180448).

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