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Influence of Polymorphism within the Heme Oxygenase-I Promoter on Overall Survival and Transplantation-Related Mortality after Allogeneic Stem Cell Transplantation

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Aside from major and minor histocompatibility antigens, genetic polymorphisms of various donor and host genes have been found to be risk factors for graft-versus-host disease and transplantation-related mortality (TRM). The heme oxygenase I (HO-I) protein has been implicated in regulating inflammatory response and has been described as a "protective gene" in solid organ transplantation. In humans, the promoter region displays length polymorphism due to a variable number of GT repeats. Individuals exhibiting 29 or fewer GT repeats express higher levels of HO-I on cellular stress compared with individuals with 30 or more GT repeats. We retrospectively analyzed length polymorphisms of 92 donor–host pairs undergoing allogeneic stem cell transplantation. Our findings demonstrate that mainly donor polymorphism leading to high expression of HO-1 (<30 GT repeats) on stress signals is associated with reduced overall survival, and that TRM is significantly increased in this group. This reduction in survival was most prominent when unrelated donors were used. Polymorphisms of the recipient HO-1 genes did not influence posttransplantation outcomes. We conclude that HO-1 polymorphism represents a new genetic risk factor for TRM and overall survival.

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KEY WORDS: Heme oxygenase, Graft-versus-host disease, Polymorphism, Stem cell transplantation, Risk factor

INTRODUCTION

Allogeneic stem cell transplantation is an important therapeutic option for various malignant and nonmalignant diseases. To date, its use has been severely limited because of transplantation-related complications leading to high mortality. Number one among these is acute graft-versus-host disease (GVHD), which continues to be the leading cause of early mortality despite the development of various new, powerful immunosuppressive drugs. Aside from the major histocompatibility complex (MHC), numerous genetic non-MHC risk factors for acute GVHD have been identified [1-4]. Most of these genetic differences involve proinflammatory cytokines and proteins regulating immune responses toward pathogens.

The pathophysiology of acute GVHD is believed to involve three steps [5-7]. To allow the donor cells to engraft, the host is preconditioned using total body irradiation and/or chemotherapy. This treatment results in the release of proinflammatory cytokines from damaged tissue, activation of host antigen-presenting cells [8], and severe impairment of the intestinal mucosal barrier. In a second step, activated host antigen-presenting cells stimulate mature alloreactive donor T cells, which then mediate damage in target tissues, such as the skin, liver, and gut. In a third, parallel step, leakage of bacterial lipopolysaccharides across the damaged mucosal barrier further

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stimulates T cells and promotes the release of proinflammatory cytokines from antigen-presenting cells [9,10].

In the pathophysiologic context of acute GVHD, the enzyme heme oxygenase I (HO-1) may be involved in several steps. HO-1 is the rate-limiting enzyme in the first step of heme degradation to biliverdin, free iron, and carbon monoxide. It represents the inducible form of 2 isoforms of HO and is expressed mainly in the spleen, where its substrate heme is abundant because of erythrocyte sequestration. Aside from heme degradation, HO-1 also has been attributed several regulatory functions in tissue inflammation and protection against stress-induced apoptosis. In fact, the protein was initially described as heat shock protein 32 (Hsp32) [11,12] and appears to play an important role in experimental solid organ transplantation [13-16]. To date, few studies have addressed the function of HO-1 in the context of experimental stem cell transplantation models. Woo et al. [17] described the effect of inducing HO-1 in donor cells before transplantation on reducing GVHD after transplantation into haploidentical hosts. Recently, our group [18] addressed the effect of HO-1 induction in the host before conditioning and showed that GVHD was significantly reduced and survival improved in animals with systemically induced HO-1 induction using cobalt protoporphyrine IX. We hypothesized that HO-1 induction will lead to tissue protection from total body irradiation–induced damage and reduce cytokine release by host antigen-presenting cells.

The human HO-I promoter contains a variable (GT)n polymorphism in the 5́ region of the promoter. GT repeats, the most frequent form of nucleotide repeats, are presumably negative gene regulators due to the formation of Z-loops [19]. The number of GT repeats seems to be critical for the formation of Z-loops and thus has an adverse influence on gene regulation in many eukaryotic and prokaryotic genes. The distribution of the number of GT repeats within the HO-1 promoter permits the grouping into short (< 25 GT repeats), medium (25 to 30 GT repeats), and long (>30 GT repeats) alleles. Short alleles are associated with greater expression on cellular stress [20-22], whereas long alleles are not. The exact mechanism of this effect remains unclear, but numerous investigators have suggested a strong role of HO-1 in oxidative stress-mediated diseases and inflammation. Given the experimental data and data from studies in humans, we hypothesized that polymorphism of the HO-1 gene promoter would influence survival and the severity of GVHD as a non-MHC risk factor. We show here that short and medium (25 to 30 GT repeats) donor alleles had an adverse effect on overall survival (OS), transplantation-related mortality (TRM) and GVHD, and that this effect was most pronounced when unrelated donors were used. These data suggest that HO-1 may represent a new non-MHC risk factor for TRM and OS.

MATERIALS AND METHODS

Patients

This study uses data from 92 patients admitted to the University of Regensburg Hospital's transplant unit for allogeneic stem cell transplantation between 1998 and 2002. The conditioning regimen was either standard intensity, using 8 to 12 Gy of total body irradiation and high-dose cyclophosphamide (61 patients), or reduced-intensity cyclophosphamide (RIC) using fludarabine, 1,3-bis(2chloroethyl)-1-nitrosurea (BCNU), and melphalan (31 patients). Grafts were from a matched related donor (MRD) in 42 patients and from an unrelated donor (URD) in 50 patients. Patients receiving grafts from URDs received additional in vivo T cell depletion by antithymocyte globulin (ATG) or, in 1 case, Campath before transplantation. Our sample did not include patients with disparate HLA donor–host pairs. Immunosuppression comprised standard cyclosporine and methotrexate or cyclosporine and mycophenolate-mofetil. The patients' underlying disease and status at the time of transplantation are shown in Table 1. Early/intermediate disease stage was defined as the first or second complete remission (CR) of acute leukemia or a chronic or accelerated phase of chronic leukemia. The late disease stage group comprised patients who did not achieve CR or were beyond the second CR. The main indications for transplantation were acute leukemia, chronic myelogenous leukemia, and non-Hodgkin lymphoma. GVHD was graded according to the Glucksberg criteria [23] and major outcome variables, such as TRM and OS, were updated monthly. The median patient age was 45 years (range, 16 to 65 years).

DNA Sample Collection and Informed Consent

DNA samples used for this study were collected from 108 consecutive patients who underwent transplantation at Regensburg between 1998 and 2002. Only 92 samples of donors and hosts (85.1%) were included, because of missing samples from either donor or host or poor-quality material. Samples were frozen before transplantation and retrospectively analyzed for HO-1 polymorphism. DNA from donor and host was prepared from peripheral blood stem cells (PBSCs) using standard procedures and kits according to the manufacturers' protocols. Informed consent by donor and host was obtained before stem cell collection for assessment of genetic risk factors for GVHD, TRM, and single nucleotide polymorphisms of inflammatory proteins. The informed consent was approved by the

Table 1. Major Characteristics of Patients and Donors and Transplantation Procedures

Patient characteristics	
Age at transplantation, years	
Mean	44.34
Median	45.00
Range	16-65
Sex	
Male	40
Female	52
Cytomegalovirus status	
Positive	41
Negative	51
Disease	
Acute myelogenous leukemia/MDS	33
Acute lymphoblastic leukemia	5
Chronic myelogenous leukemia	19
OMF	7
Non-Hodgkin lymphoma	19
Chronic lymphocytic leukemia	1
MM	4
M. Hodgkin	2
SAA/PNH	1
Mamma-CA	1
Disease stage	
Early/intermediate	54
Late	38
Conditioning regimen	
Standard	61
RIC	31
T cell depletion	
None	37
ATG/Campath/CD34	55
Donor characteristics	
Age at transplantation, years	
Mean	40.66
Median	39.00
Range	17-66
Sex	
Male	55
Female	37
Cytomegalovirus status	
Positive	32
Negative	60

Note. Standard conditioning regimens consisted of 8 to 12 Gy total body irradiation and high-dose cyclophosphamide. RIC was based mainly on fludarabine, BCNU, and melphalan. ATG and Campath were given in the course of pretransplantation conditioning.
MDS indicates myelodysplastic syndrome; SAA, severe aplastic anemia; Mamma-CA, mamma carcinoma; RIC, reduced intensity conditioning; ATG, antithymocyte globuline.

Ethics Committee of the University of Regensburg (number 02/220).

Determination of Number of (GT)n Repeats within the HO-1 Promoter Region

To determine the number of GT repeats within the promoter region of the HO-1 gene, a sense primer, 5́-GAA GAT CTT GCC AAG CAG TCA GCA GAG GAT-3́, and a fluorescein-labeled antisense primer, 5́-ACA GCT GAT GCC CAC TTT CT-3́, were used to amplify 200 ng of genomic DNA derived from PBSCs. The primers were obtained from Metabion (Martinsried, Germany). Optimal cycle conditions were annealing at 60°C for 30 seconds, followed by extension to 72°C for 60 seconds over 48 cycles. To determine fragment length, the polymerase chain reaction product was loaded on a POP-4 matrix (Applied Biosystems, Darmstadt, Germany), and calculation of fragment length was laser-based through an automated sequencer through comparison to sequenced alleles of different lengths. The range of error was < 0.5 bp.

HO-1 (GT)n Polymorphism Grouping

The number of (GT)n repeats varied from 16 to 38. We followed Yamada et al. [20] in our choice of classification by dividing the alleles into 3 groups: class S alleles, with < 25 GT repeats; class M alleles, with 25 to 29 GT repeats; and class L alleles, with ^{3} 30 GT repeats. This classification scheme affords 6 possible genotypes within the donor and host population: S/S, S/M, M/M, M/L, LL, and S/L. Based on their functional data, Yamada et al. [20] further aggregated these donor–host permutations with respect to L class alleles. Group I contains all individuals with no class L alleles (ie, only M and S alleles), and group II contains all individuals with 1 or 2 class L alleles. Thus, each patient/donor pair has 4 possible permutations: donor group I/host group I, donor group II/host group I, donor group I/host group II, and donor groupII/host group II. We followed Yamada's classification, labeling his "group I" HO-1^{high} and his "group II" HO-1_{low}. In a further categorization, we grouped patients in which neither the donor nor the host has any class L allele as HO-1^{high}, with all other permutations relegated to the second group.

Clinical and Statistical Analysis

For this study, SPSS statistical analysis software, version 14.0 (SPSS Inc, Chicago, IL) was used. Major outcome variables, including OS, TRM, overall acute GVHD, and gastrointestinal GVHD were analyzed in relation to host and donor both individually and in groups, as described earlier. The time to clinical event was calculated from the date of transplantation. For OS and disease-free survival (DFS), the Kaplan-Meier methodology was used. For overall acute GVHD and gastrointestinal GVHD, events occurring until day 100 were included. Deaths from relapse were calculated as competing risks where indicated.

RESULTS

Allele Frequency of GT Repeats among Donor and Host Groups

The number of (GT)n repeats among the individuals in our study ranged from 15 to 38. Because each individual carries 2 alleles, 184 alleles were analyzed for both donors and hosts. The histograms shown in Figure 1 show the distribution of number of GT repeats for the study population. The mode of the distribution among both donors and recipients was 29, with additional peaks at 23 and 30. The mean of the host

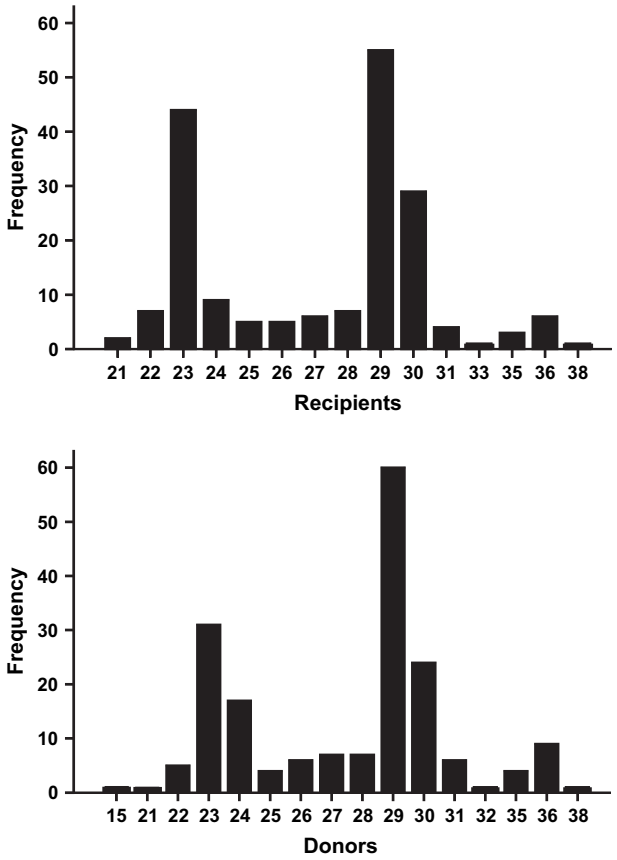

Figure 1. Frequency of number of (GT)n repeats. Each individual carries 2 alleles; thus, 184 alleles were analyzed for both the donor and host groups. The number of (GT)n repeats was analyzed as described for donors and recipients. The average number of (GT)n repeats was 27.7 ± 3.8 in the donor group and 27.3 ± 3.6 in the recipient group . The median was 29 in both groups, ranging from 15 to 38 in the donor group and from 21 to 38 in the host group. Similarity between both groups was determined using the Kolmogorov-Smirnov test ($P = .574$; $z = 0.782$).

and donor distributions was 27.3 (± 3.7) and 27.7 (± 3.8), respectively. Using a Kolmogorov-Smirnov test, we were unable to reject the null hypothesis that the donor and host allele distributions are identical ($P = .574$; Kolmogorov-Smirnov $z = 0.782$). These results are in line with data from cohorts in Japan and Europe published previously [20,24,25].

Table 2 summarizes various patient and treatment characteristics, disaggregated by group. In column 1, patients are grouped into 2 categories: those with a donor with no L-allele (≥ 30 GT repeats), the HO-1^{high} expressers, and those with 1 or 2 L-alleles, the HO-1_{low} expressers. The first 3 columns correspond to groups disaggregated by donor HO-I expression, whereas the last 3 columns correspond to patient HO-I expression. As the *P* values indicate, there is no significant difference in outcome covariates across these groups. Only in the case of sex and donor variables in the donor grouping (Table 2, donor column) is a trend toward significance seen, with males more likely to belong to the HO-1^{high} group ($P = .05$) and matched URDs more likely to belong to the HO-1_{low} group ($P = .07$). Because being male is considered a risk factor for TRM and GVHD, this overrepresentation of males could be a potential source of bias toward worse outcomes in the group of HO-1^{high} donors. In contrast, the HO-1^{high} donor group contained more matched related sibling donors, which may be a source of downward bias in GVHD development and TRM. In general, the covariates do not appear to be systematically correlated with the HO-1 grouping.

Overall Survival, Transplantation-Related Mortality, and Graft-versus-Host Disease

Table 3 describes the proportion of patients in each of the groups with respect to various posttransplantation outcomes. As the "donor" column indicates, patients receiving grafts from donors displaying an HO-1^{high} polymorphism had a significantly reduced OS ($P = .023$) and DFS ($P = .018$), as well as a trend toward significance with respect to higher TRM ($P = .057$). In contrast, polymorphism of the host had no significant influence on any of these outcomes. Only in the case of transplantation-related complications in the form of idiopathic pneumonia syndrome (IPS)/acute respiratory distress syndrome (ARDS)/pneumonia was there a trend toward significance, with HO-1^{high} polymorphism in the host associated with worse outcomes ($P = .066$). Interestingly, a combination of HO-1^{high} expressing donors with HO-1^{high} expressing hosts indicates that the absence of an L-allele in both donor and host is even more closely associated with worse transplantation-related outcomes in all but one of the outcomes considered here. Patients exhibited significantly higher TRM ($P = .006$), gut GVHD ($P = .052$), and acute severe GVHD ($P = .008$) and lower DFS ($P = .002$) and OS ($P = .011$) when both they and their hosts had HO-1^{high} polymorphism (data not shown).

Figure 2 scrutinizes the survival data for donor and host HO-1 polymorphism in more detail. It examines OS between groups disaggregated by MRDs (A) and matched URDs (B). Among the patients with MRDs, OS did not differ significantly based on this HO-I grouping (71.3% vs 55.1%). In contrast, OS in patients receiving grafts from an HO-1^{high} URD was considerably lower than that in their HO-1_{low} counterparts (41.7% vs 81.8%; $P = .015$; log-rank test). This suggests that URD polymorphism influences OS. The same cannot be said of recipient HO-1 polymorphism receiving grafts from an URD; although patients with HO-1^{high} polymorphism had lower OS than their HO-1_{low} counterparts (73.7% vs 54.8%), this difference was not statistically significant (data not shown). To support this finding, we also analyzed OS comparing HO-1^{high} MRDs and URDs and HO-1_{low} MRDs and URDs (data not shown). In neither case was any

Table 2. Distribution of Factors Influencing Outcome

	Donor			Host		
	HO-1^{high}	HO-1$_{low}$	*P*	HO-1^{high}	HO-1$_{low}$	*P*
Patient sex						
Female	20	20	.05	24	16	.57
Male	37	15		31	21	
Recipient age						
< 40 years	20	12	.56	21	11	.27
> 40 years	37	23		34	26	
Disease stage						
Early/intermediate	32	22	.34	31	23	.37
Advanced	25	13		24	14	
Donor						
Matched sibling	30	12	.07	24	18	.39
Matched unrelated	27	23		31	19	
Disease						
Acute leukemia	20	18	.40	22	16	.86
Chronic myelogenous leukemia, OMF, SAA	19	8		16	11	
Non-Hodgkin lymphoma, Hodgkin lymphoma	17	9		16	10	
Mamma carcinoma	1	0		1	0	
Sex mismatch						
Female into male	10	6	.60	30	7	.48
Others	47	29		46	9	
T cell depletion						
ATG, Campath	31	24	.13	35	20	.24
No	26	11		20	17	
Stem cell source						
Bone marrow	19	7	.13	15	11	0.49
PBSC	38	28		40	26	
Conditioning						
Standard	35	26	.15	39	22	0.18
RIC	22	9		16	15	

Note. Donor and host were grouped according to number of (GT)n repeats, as described in Materials and Methods. There was a trend toward significance with more male patients in the HO-1^{high} donor group ($P = 05$) and more transplantations from matched siblings in the HO-1^{high} donor group ($P = 07$).
OMF indicates osteomyelofibrosis; SAA, severe aplastic pneumonia.

statistically significant difference observed (HO-1^{high} MRD vs URD, $P = .167$; HO-1$_{low}$ MRD vs URD, $P = .705$). When HO-1 polymorphism groups were disaggregated according to the use of ATG as in vivo T cell depletion, we observed similar results with respect to donor polymorphism, because most ATG recipients are in the URD group (OS: no ATG/Campath HO-1^{high}, 64.9% vs HO-1$_{low}$, 72.7%, $P = .593$; ATG/Campath HO-1^{high}, 48.4% vs HO-1$_{low}$, 83.3%, $P = .012$; TRM: no ATG/Campath HO-1^{high}, 20.0% vs HO-1$_{low}$, 9.1%, $P = .463$; ATG/Campath HO-1^{high}, 35.5% vs HO-1$_{low}$, 12.5%, $P = .041$).

Table 3. Transplantation-Related Mortality, Graft-versus-Host Disease, Disease-Free Survival, and Overall Survival

	Donor			Host		
	HO-1^{high}	HO-1$_{low}$	*P*	HO-1^{high}	HO-1$_{low}$	*P*
TRM	28.1	11.4	.057	25.5	16.2	.318
Gut GVHD ≥ 3	17.5	5.7	.111	16.4	8.1	.349
GVHD ≥ 3	28.1	14.3	.123	29.1	13.5	.127
IPS, ARDS, pneumonia	23.8	33.3	1.000	37.5	0.0	.066
DFS	45.6	71.4	.018	49.1	64.9	.19
OS	54.4	80.0	.023	60.0	70.3	.61

Note. All values are given as percentages. Significance was calculated using the χ^2 test for acute GVHD, gut GVHD, and IPS; for OS, TRM and DFS was calculated using the log-rank test.

Figure 3 shows the incidence of TRM (A) and severe acute GVHD (B) using URDs only. As depicted, TRM was increased significantly when HO-1^{high} expressing URDs were used (48.5% vs. 14.03%; $P = .030$; log-rank test). Similarly, the incidence of severe acute GVHD in this group also was higher, but the difference did not reach statistical significance at the 5% level (37.7% vs. 18.5%; $P < .08$; log-rank test).

Influence of Conditioning and Disease-Free Survival

Based on our experimental data, we can conclude that HO-1 expression can confer protection against irradiation-induced damage and consequent GVHD. Given the regulatory potential of HO-1 with respect to apoptosis and anti-inflammatory properties, we analyzed the impact of conditioning regimens on outcomes in different polymorphism subgroups. Our results pertaining to OS and TRM given earlier were not affected by the conditioning regimen applied; neither standard nor reduced intensity conditioning influenced the differences seen within the donor HO-1 polymorphism. Similar results also were obtained with respect to DFS. As shown in Table 3, HO-1^{high} donor polymorphism was associated with reduced DFS, which was somewhat surprising, because more

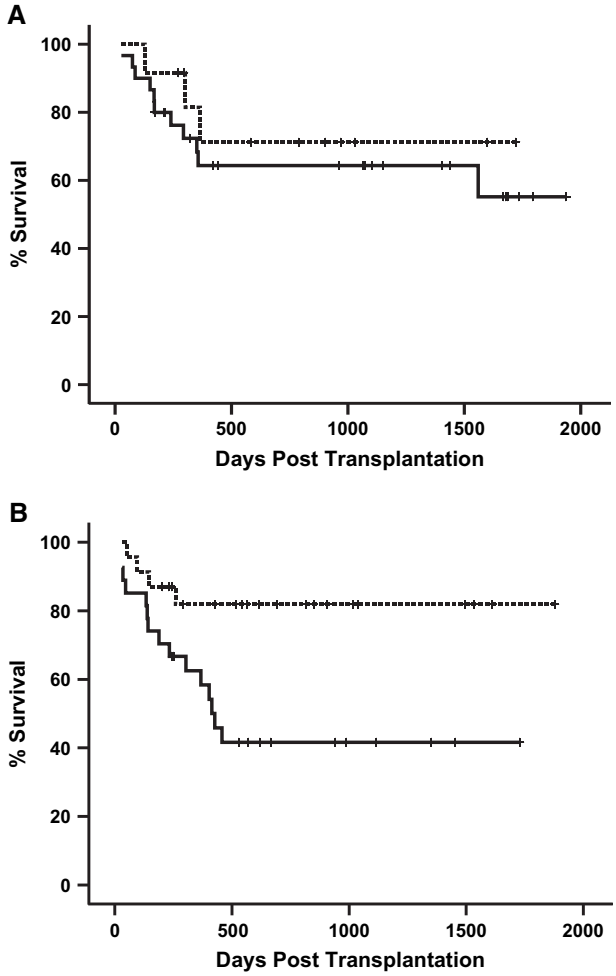

Figure 2. OS of donor HO-1^{high} donors (solid line) and HO-1$_{low}$ donors (dotted line). A, OS using MRDs (HO-1^{high}, 71.3% vs HO-1$_{low}$, 55.1%; P = not significant; log-rank test). The number of events/number of patients was 11/29 for HO-1^{high} and 3/11 for HO-1$_{low}$. The median GT(n) repeats was 29, and the average was 27.3 ± 4.3. B, OS using matched URDs (HO-1^{high} 41.7% vs HO-1$_{low}$ 81.8%; P = .015; log-rank test). The number of events/number of patients was 14/26 for HO-1^{high} and 4/22 for HO-1$_{low}$. The median GT(n) repeats was 29, and the average was 27.4 ± 3.4.

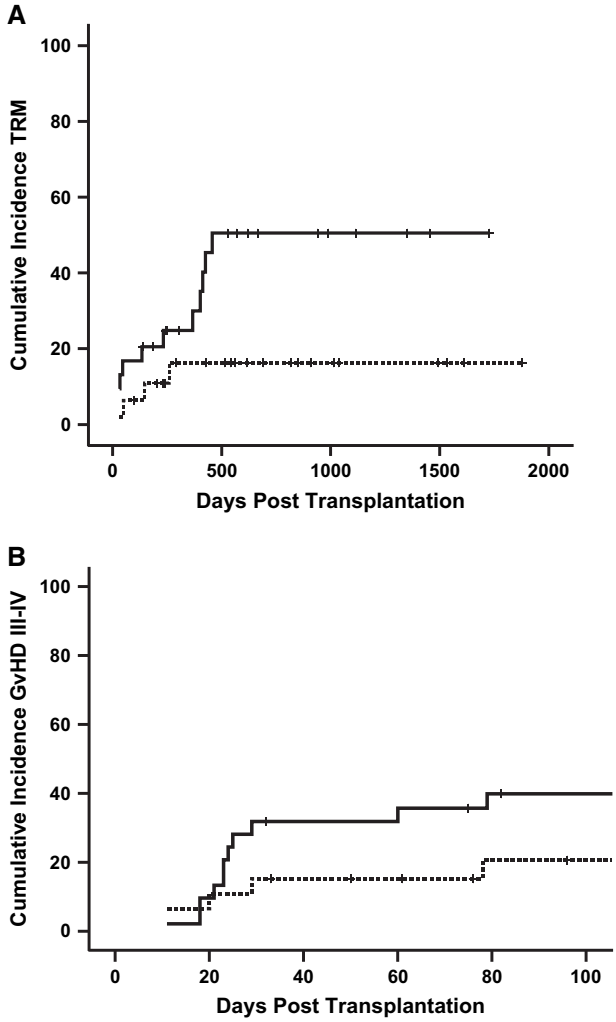

Figure 3. TRM and acute severe GVHD. HO-1^{high} donors (solid line) compared with HO-1$_{low}$ donors (dotted line) using only URDs. A, TRM was increased in the group of unrelated HO-1^{high} donors (48.5% vs 14.3%; P = .030; log-rank test). The number of events/number of patients was 11/26 for HO-1^{high} and 3/22 for HO-1$_{low}$ 3/22. B, Cumulative incidence of acute severe GVHD (grade 3-4) was increased when HO-1^{high} URDs were used (37.7% vs 18.5%; P < .08; log-rank test). The number of events/number of patients was 8/26 for HO-1^{high} and 4/22 for HO-1$_{low}$.

severe GVHD also was seen in that same group. Comparing DFS in the MRD and URD groups revealed no difference in outcomes (57.1% in MRD vs 54% in URD; P = .541); however, HO-1 polymorphism had an effect on DFS in the URD group but did not in the MRD group. In the former group, HO-1^{high} donor polymorphism negatively influenced DFS (HO-1^{high}, 46.3% vs HO-1$_{low}$, 74.6%; P = .120). Host polymorphism had no influence on DFS (HO-1^{high} 56.2% vs HO-1$_{low}$ 71.1%; P = .521). As shown in Figure 4A, when HO-1 polymorphism of donor and host were grouped such that HO-1^{high} expressing donors and recipients formed a single group against all other permutations containing at least 1 HO-1$_{low}$ allele, again no significant difference in DFS was seen when MRDs were used; however, in the URD group, DFS was significantly reduced (HO-1^{high} donor and host, 32.1% vs all other permutations, 76.1%; P = .002). Almost superimposable results were obtained when data were analyzed according to the use of ATG in the conditioning regimen, because with few exceptions, only patients with a matched URD received ATG for conditioning.

Multivariate Analysis of Risk Factors Associated with Transplantation-Related Mortality and Overall Survival

Table 4 presents results from our Cox regression analyzing competing risk factors for OS and TRM for URDs. T cell depletion by ATG or Campath was not included as a risk factor, because in almost all cases, patients receiving grafts from URDs received ATG,

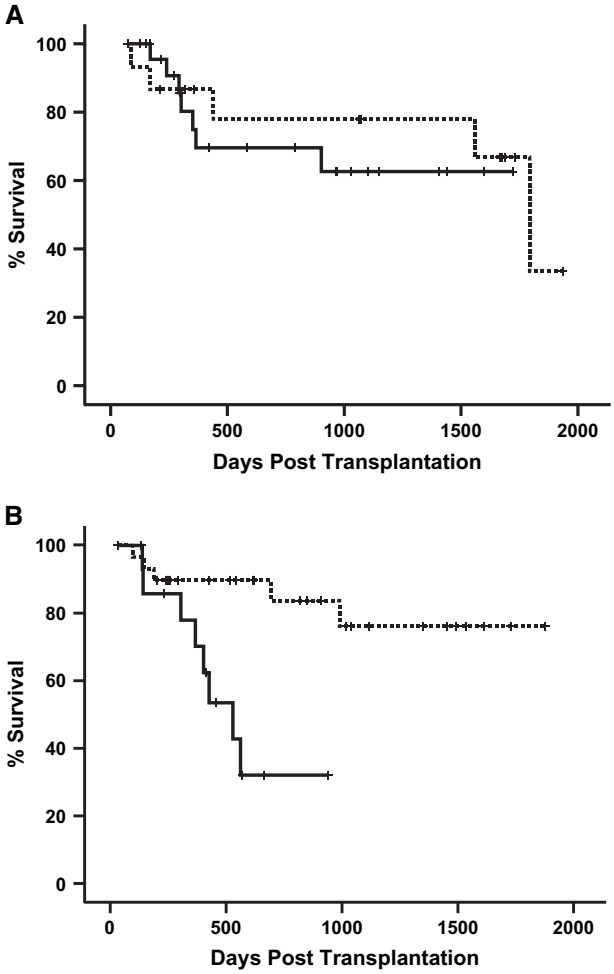

Figure 4. DFS of donor/recipient HO-1^{high} groups (solid line) compared with all other permutations of donor and host HO-1 polymorphisms containing at least 1 HO-1$_{low}$ allele of donor or recipient (dotted line). A, DFS using MRDs. No significant difference was observed (62.6 vs. 33.4, $P = .582$) B, DFS using URDs (HO-1^{high} donor and host, 32.1% vs all other permutations, 76.1%; $P = .002$).

and in 1 case, Campath, during the conditioning regimen (MRD, 6 of 42 vs URD, 49 of 50; $P < .001$; χ^2 test). The HO-1 polymorphism grouping in the last row pertains to that of the URD. As the *P* values for OS indicate, only the disease stage and donor HO-1 polymorphism had a significant impact on OS. Patients with advanced disease are 3.01 times more likely to die than those at an early or intermediate stage ($P = .021$). HO-1^{high} donor polymorphism also verged on significance for both OS ($P = .029$) and TRM ($P = .026$); indeed, in the case of TRM, HO-1^{high} donor polymorphism was the only significant risk factor. Interestingly, when we ran an analogous Cox regression, substituting HO-1^{high} host polymorphism for HO-1^{high} donor polymorphism, we found that this variable had no significant effect on either OS ($P = .87$) or TRM ($P = .62$) (data not shown). Because after transplantation, 2 possible genotype groups are combined, we also included in our Cox regression model the genotype of the recipient as a competing risk factor

Table 4. Multivariate Risk Factor Analysis for OS and TRM According to HO-1^{high} Donor Polymorphism

	n	Number of Events	HR	95% CI	*P*
OS					
Age					
≤ 40 years	24	10	1.00	0.38–2.34	.904
> 40 years	26	9	0.95		
Disease stage					
Early/intermediate	28	6	1.00	1.13–8.05	.021
Advanced	22	13	3.01		
Stem cell source					
Bone marrow	15	5	1.00	0.33–2.89	.967
PBSC	35	14	0.97		
HO-1 polymorphism					
Donor HO-1$_{low}$	23	4	1.00	1.02–9.52	.029
Donor HO-1^{high}	27	15	3.12		
TRM					
Age					
≤ 40 years	24	9	1.00	0.17–1.57	.239
> 40 years	26	5	0.53		
Disease stage					
Early/intermediate	28	6	1.00	0.60–5.34	.284
Advanced	22	8	1.80		
Stem cell source					
Bone marrow	15	4	1.00	0.26–3.14	.881
PBSC	35	10	0.90		
HO-1 polymorphism					
Donor HO-1$_{low}$	23	4	1.00	1.04–13.4	.026
Donor HO-1^{high}	27	10	3.73		

Note. Age, disease stage, stem cell source, and HO-1 polymorphism of donor and host were included in the analysis as relevant risk factors for OS and TRM. Only patients with unrelated donors were included, and thus all patients underwent T cell depletion by ATG or Campath during conditioning.

for URDs. The qualitative results were unchanged. For TRM ($P = .043$; hazard ratio [HR] = 3.73; 95% confidence interval [CI] = 1.04 to 13.4), donor genotype remained the only significant risk factor. For OS, HO-1^{high} donor polymorphism was significant ($P = .045$; HR = 3.12; 95% CI = 1.02 to 9.53), and, as expected, disease stage remained a significant risk factor ($P = .027$' HR = 3.02, 95% CI = 1.13 to 8.06).

To prevent the introduction of bias by choosing matched URD transplants only for the Cox regression analysis and neglecting the interaction between transplant type and genotype, we also analyzed the influence of donor and host genotype in the entire cohort (data not shown). Similar to the foregoing results, donor polymorphism verged on significance as a risk factor for OS ($P = .064$; HR = 2.21; 95% CI = 0.95 to 5.11) as well as for TRM ($P = .037$; HR = 3.25; 95% CI = 1.08 to 9.83).

DISCUSSION

Over the last decade, the HO-I system has emerged as a major factor in controlling local inflammation and inducing tolerance [26,27]. So far, the effects of this protein have been reported in experimental systems with regard to solid organ transplantation [28,29]. In addition, there have been numerous reports on the involvement of polymorphisms within the promoter

region of the human HO-1 gene in various diseases of the pulmonary and vascular systems [20,30]. To the best of our knowledge, this is the first report analyzing the impact of HO-1 polymorphisms in an allogeneic stem cell transplantation setting.

Using retrospective data on 92 patient–donor pairs, we find that length polymorphism in the donor but not in the host alone significantly influence such posttransplantation outcomes as OS and TRM. In particular, patients in our data with medium and short alleles [< 30(GT)n repeats] in the donor alone typically have significantly worse outcomes.

The number of (GT)n repeats within the promoter region influences the expression levels of HO-1 on cellular stress. Short alleles [< 30 (GT)n repeats] are associated with the ability to mount a quantitatively higher HO-1 response. Our finding is thus at odds with a number of other studies that ascribe a protective role for high HO-1 expression (reviewed by Exner et al. [31]). Studies in humans as well as experimental studies in solid organ transplantation have suggested that expression of HO-1 in specific tissues is generally associated with prolonged graft survival, reduced leukocyte infiltration, and, more recently, even with tolerance [28,29,32-34]. Results based on our retrospective data also appear to run counter to those based on experimental data from our group and others [17,18]. In an earlier experimental study, we showed that induction of HO-1 in recipient mice using protoporphyrins as an HO-1 inductor is associated with improved OS, reduced GVHD and reduction of the release of proinflammatory cytokines. Obtaining similar results by administering protoporphyrins to the donor, Woo et al. [17] argued that donor cells rather than the recipient are influenced by the induction of HO-1. Due to the antiapoptotic effects of HO-1, we hypothesized in our experimental study that induction of HO-1 before conditioning would protect the host and thereby reduce GVHD. This hypothesis was confirmed by histology and reduced lipopolysaccharide transfer across the damaged gut mucosa. What the data presented here suggest, however, is that the host's ability to mount an HO-1 response has little or no bearing on posttransplantation outcomes. One possible explanation for this discrepancy is that we do not yet know whether the experimental data on generally high HO-1 expression (vs no expression in untreated mice) within parenchyma reflects the differences in expression seen in humans due to the variable number of (GT)n repeats. In addition, HO-1 expression in donor cells is associated with improved survival [17], in contrast to the results in this study, where HO-1^{high} expressing donors contribute to significantly to worse outcomes.

At this stage, it is worth making a number of qualifications regarding our findings. First, our data are retrospective, and the sample size is limited. Second, although we do take well-established risk factors relating to posttransplantation outcomes into account, the specification of HO-1 in the empirical model, although based on data from other studies, remains speculative; for example, given the distribution of (GT)n repeats (Figure 1), our results are clearly sensitive to the specification of the threshold number of (GT)n repeats for HO-1_{low} or HO-1^{high} expression. Therefore, we conducted some robustness checks, varying the threshold above and below the 30 GT repeats used by Yamada et al. [20] and in the present study. For all thresholds above 30 and all those below 30, there was no statistical difference in OS and TRM between HO-1_{low} or HO-1^{high} under the new categorization. In essence, by shifting the threshold down, we moved SS, SM, or MM alleles (HO-1^{high}) into the L allele (HO-1_{low}) group. This worsened the outcomes in this group. The same logic held in reverse when we moved thresholds upward. Given that our distribution of (GT)n repeats is in line with published data, these robustness checks support the use of this threshold. In addition, the grouping of our data into HO-1^{high} and HO-1_{low} was necessitated by the fact that each patient has 2 alleles. Both alleles have been shown to play a role in HO-1 expression, but the question remains as to which length polymorphism is driving the divergent outcomes in the 2 groups. To examine this, we excluded mixed genotypes of the donors (ML and SL) from the analysis and compared only HO-1^{high} groups (SS, SM, and MM) with HO-1_{low} groups (LL). Doing so led to an even more pronounced difference in outcomes; Kaplan-Meyer estimates indicated an OS of 100% in the HO-1_{low} LL group and 45.8% in the HO-1^{high} group (P = .012, data not shown) and corresponding TRMs of 0% and 34.4% (P = .047; data not shown). This suggests that the presence of an extra L allele may play an important protective role posttransplantation.

One striking observation in our study was the effect of URD HO-1^{high} polymorphism on OS and TRM. The clear difference between the MRD and URD groups in our cohort is the use of in vivo T cell depletion before transplantation by ATG or Campath. When MRDs and URDs were included in the multivariate analysis, T cell depletion per se had no impact on the outcome (data not shown). The link between T cell function and its modulation by HO-1 expression (or vice versa) within parenchyma or in professional antigen-presenting cells is poorly understood. There is increasing data on how Tregs manipulate the parenchymal environment and thereby down-modulate inflammatory responses [35,36]. One proposed model describes the induction of inducible nitric oxide synthase and indolamine-2-3-dioxygenase by interferon (IFN)-γ derived from activated Tregs and subsequent release of 3-hydroxyanthranilic acid, which is a strong inducer of HO-1 [37]. Activation

of HO-1 in parenchyma and monocytes/macrophages then counterbalances the excessive release of proinflammatory cytokines and provides a survival signal for target tissue cells. The depletion of Tregs by ATG and Campath may lead to a missing anti-inflammatory stimulus and an interrupted negative feedback loop. One interpretation would be that individuals with HO-1^{high} polymorphism are more dependent on this genetically defined counterregulation. This hypothesis also would explain why the combination of donor and host HO-1^{high} polymorphisms worsens outcomes after allogeneic stem cell transplantation. In this situation, both sides are unable to properly balance their response.

The depletion of T cells through ATG or Campath is not limited to Tregs and depletion of part of the donor, and most of the host T cell compartment mainly affects the release of IFN-γ during the peritransplantation period. The function of this cytokine in the context of allogeneic stem cell transplantation remains under debate, but pleiotropic function in exacerbating GVHD and preventing GVHD has been reported in experimental mouse models (as reviewed by Yang et al. [38]). Several experimental models have demonstrated that donor-derived IFN-γ is necessary for reduction of acute GVHD [39]. In addition, polymorphisms within the human IFN-γ gene leading to lower expression (IFN-γ intron1 3/3 genotype) are associated with the development of severe GVHD [4,40]. So far, few studies have revealed a link between HO-1 expression and IFN-γ release [41-43], but those authors suggested that the protective effects of IFN-γ are associated with up-regulation of HO-1 in the parenchyma, leading to reduced immunogenicity. In line with these findings is the observation that in humans, unlike mice, IFN-γ represses HO-1 up-regulation [44]. Depletion of host T cells and parts of donor T cells using ATG before conditioning results in reduced IFN-γ release and reduced HO-1 induction. Donors and hosts with HO-1^{high} polymorphism are presumably more dependent on up-regulation of HO-1 as a means of inflammatory counteraction and this may explain the increased TRM in this case.

Using ATG in the conditioning regimen had an influence on the relapse rates when the data were disaggregated by HO-1 polymorphism. Although we observed no difference in DFS according to MRD or URD overall, the use of ATG in the URD group led to deleterious DFS survival, when both donor and recipient displayed HO-1^{high} polymorphism (Figure 4B). This corroborates findings in recent publications suggesting that the expression of HO-1 represents a survival and resistance factor for leukemic cells [21,45-47]. Polymorphism and different levels of expression also may play a role in sufficient leukemia rejection, because the largest number of severe acute GVHD was observed in this group of patients.

In summary, the data presented in this study describe a potentially novel non-MHC risk factor that significantly impacts OS, TRM, and acute and severe GVHD after allogeneic stem cell transplantation. The influence of HO-1 on the regulation of the immune system is not fully understood and requires further experimental support. The evidence presented here on the basis of a retrospective study needs to be confirmed by a prospective investigation.

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