Prognostic value of donor cytotoxic T-lymphocyte precursor frequencies for acute graft-versus-host disease in hematopoietic stem cell transplantation from HLA-matched siblings: a single center experience in a cohort of 92 patients

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We investigated the prognostic value of cytotoxic T-lymphocyte precursor frequencies (CTL-p-f) for the development of graft-versus-host disease (GvHD) in a cohort of 92 recipients of a hematopoietic stem cell transplantation from HLA-matched sibling donors. CTL-p-f and clinical variables were correlated with acute GvHD and chronic GvHD in univariate and multivariate analyses. CTL-p-f resulted an independent risk factor for severe acute GvHD. Moreover, a trend towards a correlation between CTL-p-f and chronic GvHD was observed. In summary CTL-p-f may be considered as a functional assay useful for identifying patients at high risk of severe GVHD.

Key words: hematopoietic stem cell transplantation, cytotoxic T-lymphocyte precursor frequencies, graft-versus-host disease.

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Functional assays, including the analysis of cytotoxic T-lymphocyte precursor frequencies (CTL-p-f), are performed before hematopoietic stem cell transplantation from an HLA-matched sibling donor in order to evaluate *in vitro* alloreactivity elicited by minor histocompatibility antigens, which probably promotes *in vivo* graft-versus-host disease (GvHD). In a previous study, we found that high donor (>1/10^5) CTL-p-f were significantly associated with the development of grades II to IV acute GvHD in a cohort of 51 patients receiving transplants from non HLA-matched sibling donors.[1] The assay provided important information on acute GvHD outcome, although other variables might be relevant. In fact, several risk factors for acute GvHD have been reported for HLA-matched sibling allografts, including donor/recipient characteristics and clinical variables.[2-4] In the present study we evaluated the role of high donor CTL-p-f in predicting clinically significant acute GvHD and chronic GvHD in a larger cohort of patients.

Design and Methods

Ninety-two oncohematologic patients who underwent hematopoietic stem cell transplantation from an HLA-matched sibling donor in the Transplantation Unit of S.G. Battista Hospital were included in the present study. Fifty-one patients grafted between 1996 and 2000 were included in the first study, while 41 patients who received their transplant between 2000 and 2004 constitute the most recently up-dated cohort of patients. Table 1 summarizes the patients' characteristics. Sibling donors were selected using serological typing for HLA-A, B, C and polymerase chain reaction low resolution typing for HLA-DRB1*. DNA sequence based typing for HLA-A*, B*, DRB1*, DQA1* and DQB1* alleles was used to confirm sibling identity. CTL-p-f assays were performed as previously described.[1] Briefly, donor responder peripheral blood mononuclear cells were cultured in limiting dilution with a constant number of irradiated recipient stimulator peripheral blood mononuclear cells. Negative controls were irradiated stimulator cells incubated without responders. An autologous control with donor responder cells against irradiated donor stimulator cells and a positive control with donor responder against HLA-incompatible stimulator cells were performed. Cultures were fed on day 3 with interleukin-2 and on day 7 were assayed for cytotoxic activity against ^{51}Cr-labeled OKT3-induced blasts, obtained from the original stimulator. The amount of ^{51}Cr release was evaluated by a β-radiation counter. Wells were scored as *positive* when the count was higher than the mean plus three standard deviation of the negative control. CTL-p-f were obtained using a computer program. The clinical cut-off (1 CTL-p /10^5) was chosen as previously described.[1] CTL-p-f and clinical variables were analyzed for an association with the development of both acute GvHD grades II-IV and extensive chronic GvHD; furthermore, the relationship between CTL-p-f and onset of chronic GvHD (quiescent, progressive, *de novo*) was examined. Logistic regression was used for both univariate and multivariate analyses and only factors reaching statistical significance ($p \leq 0.05$) in univariate analysis were included in the multivariate model. Data were analyzed using Statsoft Statistica 6.0 software.

Table 1. Donor and recipient characteristics compared in the two cohorts.

Characteristics	*First cohort (%) (1996-2000)*	*Second cohort (%) (2000-2004)*	*p-value°*
Number of patients	51	41	
Diagnosis of underlying disease			
Acute myeloid leukemia	16(31)	14(34)	0.7
Acute lymphoblastic leukemia	11(22)	4 (10)	0.2
Chronic myeloid leukemia	11(22)	10 (24)	0.7
Myelodysplastic syndrome	7(14)	7 (17)	0.6
NHL, HD, MM	6(12)	6 (15)	0.7
Disease Status at HSCT			
Poor Risk	23(45)	16(39)	0.5
Good Risk	28(55)	25(61)	
Donor Age, years (median)			
≥40	25(49)	22 (54)	0.6
<40	26(51)	19 (46)	
Recipient Age, years (median)			
≥40	25(49)	23 (56)	0.5
<40	26(51)	18 (44)	
Sex Mismatch (F donor/M recipient)			
Yes	11(22)	14 (34)	0.2
No	40(78)	27(66)	
Donor CMV serology*			
Positive	41(86)	33 (85)	0.9
Negative	7(14)	6 (15)	
Recipient CMV serology*			
Positive	41(86)	30 (77)	0.3
Negative	7(14)	9 (23)	
Conditioning Regimen			
TBI/Cy	25 (49)	17 (42)	0.5
Thio or Bu /Cy	26 (51)	24 (58)	
Source of stem cells			
Bone marrow	19 (37)	12 (29)	0.4
Peripheral blood stem cells	32 (63)	29 (71)	
GvHD prophylaxis			
1 mg/Kg CsA + MTX	33 (65)	11 (27)	0.0003
2 mg/Kg CsA + MTX	18 (35)	30 (73)	
CTL-p-f			
>1/10^5	21 (41)	21 (51)	0.2
≤1/10^5	30 (59)	20 (49)	
CD34+°			
≥7.0×10^6/Kg	28 (57)	18 (46)	0.3
< 7.0×10^6/Kg	21 (43)	21 (54)	
Acute GvHD incidence			
0-I	24 (47)	26 (64)	0.1
II-IV	27 (53)	15 (36)	
Chronic GvHD incidence			
Absent/Limited	34 (79)	29 (78)	0.9
Extensive	9(21)	8(22)	

*NHL: non-Hodgkin's lymphoma; HD: Hodgkin's disease; MM: multiple myeloma; TBI: total body irradiation; Thio: thiotepa; Bu: busulphan; Cy: cyclophosphamide; CsA: cyclosporine A; MTX: methotrexate; CTL-p-f: cytotoxic-T-lymphocyte precursor frequencies. Good risk disease includes acute leukemias in first remission, chronic myeloid leukemia in chronic phase and lymphoma in first or second remission. Poor risk disease includes all other diseases and stages. *No CMV data for five pairs. °No CD34+ data for four pairs. CD3+ cell dose was not compared between the two groups because of a conspicuous lack of data in the first cohort (about 50% of cases). °referred to Pearson's χ^2 test.*

Results and Discussion

Acute GvHD

Among the 92 patients, 50 (54%) developed grades 0-I acuteGvHD, 34 (37%) developed grade II and 8 (9%) grades III-IV. The incidence of acute GvHD grades II-IV was 53% in patients included in the first study (27 out of 51 patients) as compared to 36% in subjects in the second cohort of patients (15 out of 41 patients). The positive predictive value of CTL-p-f decreased from 71% in the first cohort to 48% in the second cohort. Overall, sex mismatch and high CTL-p-f were significantly associated with the development of grades II to IV acute GvHD in univariate analysis (Table 2). These variables were entered into the logistic regression model and only CTL-p-f reached statistical significance as prognostic factor (p=0.04; odds ratio (OR)=2.487; 95% confidence interval (CI)=1.026-6.024). The positive predictive value of the assay in the whole cohort was 60%.

Chronic GvHD

Eighty patients who were alive and disease free on day +100 post-transplantation were evaluated for the presence of chronic GvHD.Twenty-four patients (30%) had limited chronic GvHD, 17 (21%) had extensive chronic disease, while 39 patients (49%) did not develop signs of the disease. Of the 43 evaluable patients included in the first study, 9 developed extensive chronic GvHD, as compared to 8 of 37 patients included in the second group. Seventeen patients (21%) had quiescent chronic GvHD, 21 (26%) had progressive disease and 3 (4%) developed *de novo* chronic GvHD. In univariate analysis, three factors were statistically associated with the development of extensive chronic GvHD: grades II to IV acute GvHD (p=0.03), sex mismatch (p=0.01) and CTL-p-f (p=0.01). In multivariate analysis none of these factors reached statistical significance although high CTL-p-f identified a group at higher risk of extensive chronic GvHD (p=0.07; OR=3.262; 95% CI=0.872-12.21). No correlation was found between CTL-p-f and onset of chronic GvHD, although a significant association between high CTL-p-f and extensive chronicGvHD with a quiescent onset was noted (Table 3). *In vitro* alloreactivity, promoted by mismatches between minor histocompatibility antigens, may be considered as a model of *in vivo* graft-versus-host reactions and the frequencies of donor cytotoxic T-lymphocytic precursors could provide information predicting GvHD development in recipients of HLA-matched hematopoietic stem cell transplantations from sibling donors. The results of our study showed that high CTL-p-f represent an independent risk factor for the development of severe acute GvHD. Of note, the positive predictive value was 71% in the first cohort as compared to 48% in the second cohort of patients. This difference might be explained by the fact that the proportion of patients who received high-dose cyclosporine A as GvHD prophylaxis (2 mg/Kg) in the second cohort of patients was double that in the first group (73% vs 35%, p=0.0003); accordingly, the incidence of severe acute GvHD was 36% in the second cohort of patients

Table 2. Univariate analysis of risk factors for acuteGvHD in the whole cohort.

Variables	*Patients with acuteGvHD grades II to IV*	*p-value*
Donor age, years (median)		
≥ 40	22/47	*p*=0.8
< 40	20/45	
Recipient age, years (median)		
≥ 40	21/46	*p*=0.6
< 40	21/46	
Sex mismatch (female D/male R)		
Yes	16/25	*p*=0.03
No	26/67	OR= 2.803
		CI=[1.067-7.366]
Diagnosis of underlying disease		
Acute myeloid leukemia	13/30	*p*=0.8
other	29/62	
Acute lymphoblastic leukemia	8/15	*p*=0.5
other	34/77	
Chronic myeloid leukemia	11/21	*p*=0.5
other	31/71	
Myelodysplastic syndrome	6/14	*p*=0.8
other	36/78	
NHL, HD, MM	4/12	*p*=0.4
other	38/80	
Conditioning regimen		
TBI/Cy	20/42	*p*=0.7
Thio or Bu/Cy	22/50	
ABO		
Incompatible	11/33	*p*=0.08
Compatible	31/59	
Major	7/22	*p*=0.3
Minor+Compatible	35/70	
Recipient CMV serology*		
Positive	36/71	*p*=0.2
Negative	5/16	
Donor CMV serology*		
Positive	37/74	*p*=0.2
Negative	4/13	
GvHD prophylaxis		
1 mg/Kg CsA + MTX	21/43	*p*=0.7
2 mg/Kg CsA + MTX	21/49	
CTL-p-f		
> $1/1\times10^5$	25/42	*p*=0.01
≤ $1/1\times10^5$	17/50	OR= 2.854
		CI=[1.206-6.755]
Source of stem cells		
Bone marrow	13/31	*p*=0.6
Peripheral blood stem cells	29/61	
Disease Status		
Poor risk	16/39	*p*=0.4
Good risk	26/53	
$CD34^+$ cell dose (median=7.0×10^6/Kg)°		
≥ $7,0\times10^6$/Kg	24/46	*p*=0.4
< $7,0\times10^6$/Kg	18/42	
$CD3^+$ cell dose (median=1.8*108/Kg)§		
≥ $1,8\times10^8$/Kg	15/29	*p*=0.6
< $1,8\times10^8$/Kg	13/29	

*NHL: non-Hodgkin's lymphoma; HD: Hodgkin's disease; MM: multiple myeloma; TBI: total body irradiation; Thio: thiotepa; Bu: busulphan; Cy: Cyclophosphamide; CsA: cyclosporine A; MTX: methotrexate; CTL-p-f: cytotoxic-T-lymphocyte precursor frequencies.*No CMV data for five pairs.° No CD34+ data for four pairs. §No CD3+ data for 34 pairs.*

Table 3. Types of chronicGvHD onset and relationship with CTL-p-f in whole cohort.

	Patients with high CTL-p-f (>$1/1\times10^5$)	*p-value**
Progressive chronic GvHD	12/21	0.9
Other	11/20	
Quiescent chronic GvHD	8/17	0.3
Other	15/24	
Extensive Progressive chronic GvHD	5/7	0.3
Limited Progressive chronic GvHD	7/14	
Extensive Quiescent chronic GvHD	6/8	0.04
Limited Quiescent chronic GvHD	2/9	

**p value referred to logistic regression analysis.*

as compared to 53% in the first cohort (p=0.1) leading to a probable increase of false positive results. Moreover, false positives could also be explained by the different tissue-specific expression of minor histocompatibility antigens. HA-1 and HA-2 are mainly expressed on hematopoietic cells, and promote a graft-versus-leukemia effect rather than a graft-versus-host reaction.[5] Accordingly, *in vitro* detection of HA-1 or HA-2 mismatch could generate high CTL-p-f, without clinical acuteGvHD. Even the percentage of false negatives could be a limiting factor for a stronger correlation between CTL-p-f and acute GvHD. In our series, 17 out of 50 patients (34%) with low CTL-p-f experienced severe acute GvHD. This could be explained by several factors. First, some minor histocompatibility antigens usually considered as a target of a graft-versus-host reaction, might not be expressed on peripheral blood mononuclear cells. In fact, some minor histocompatibility antigens such as CD31 peptides appear to be expressed mainly in non-hematopoietic lineages.[6] Dickinson *et al.* adopted a skin-explant model to overcome this problem and to obtain a better stimulation of responder cells.[7] Secondly, peripheral blood mononuclear cells isolated from onco-hematologic patients might not properly stimulate the activation and proliferation of donor anti-recipient specific T cells even in the presence of mismatched minor histocompatibility antigens. We, therefore, recently set up a control to evaluate the stimulation capability. In case of poor stimulation, i.e. low third party anti-recipient CTL-p-f in association with low donor anti-recipient CTL-p-f and high donor anti-third party CTL-p-f, we will consider the result not reliable: this could diminish false negative results in our patients. Thirdly, the development of acuteGvHD in patients with low pre-transplant CTL-p-f, could be due to factors other than minor histocompatibility antigen revealed by functional assay, including cytokine gene polymorphism.[8] In univariate analysis, CTL-p-f acute GvHD and sex mismatch were associated with the presence of extensive chronic GvHD, while in multivariate analysis none of these factors reached a statistically significant level. Hence, we examined the relationship between CTL-p-f and the onset of chronic GvHD:

a significant correlation was found between high CTL-p-f and extensive chronicGvHD with quiescent onset. This could indicate that high CTL-p-f identified extensive chronic GvHD arising from previous acuteGvHD episodes, but our results may have been influenced by the small number of patients studied and therefore should be considered suggestive rather than definitive.

In summary, the results of the present study suggest that the CTL-p-f assay may be considered useful for identifying patients at high risk of severe acute GvHD. Nevertheless, the positive predictive value of the assay was influenced by cyclosporine A prophylaxis, which probably reduced the incidence of acute GvHD. It could be hypothesized that genetic differences revealed by *in vivo* functional assay could be less important for the development of acute GvHD *in vivo* in the presence of higher immunosuppressive treatment. For this reason, in the near future we will try to develop further strategies to predict acute GvHD, particularly in the immediate post-transplant phase.This could allow a better definition of the immunologic status of the recipients after allograft procedures.

FS was responsible for the design of the study, CTL-p-f assays, and writing the manuscript; PM performed the statistical analysis; FL, AB, MF were responsible for transplantation procedures, follow-up of patients, collection of clinical data and reviewing the manuscript; PA set-up the CTL-p-f assay and performed the initial study; GM performed HLA typing; A-MDO and AA reviewed the manuscript. The authors declare that they have no potential conflicts of interest.

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