Association of HLA-C Disparity With Graft Failure After Marrow Transplantation From Unrelated Donors

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Disparity for HLA-A or HLA-B antigens increases the risk of marrow graft rejection, but the relevance of HLA-C is unknown because typing methods have not been sufficiently accurate for clinical use. We designed a matched case-control study and employed DNA sequencing methods to evaluate the role of HLA-C disparity in 21 patients who experienced graft failure (cases) following transplantation with unmanipulated marrow from either HLA-A, B serologically matched, DRB1 matched (n = 14) or single locus mismatched (n = 7) unrelated donors. For each case, two patients who successfully engrafted were selected as controls based on similarity for factors known or suspected to influence engraftment. The estimated odds ratio (OR) of graft failure for an HLA-C mismatch relative to match (univariable model) was 5.2 (95% CI, 1.4, 19; P = .01). Serologically undetectable HLA-A or HLA-B allele disparity was also associated with graft failure. The association between HLA-C disparity and graft failure remained significant even after accounting for the contribution of HLA-A and/or HLA-B allele disparity (OR 4.0; 95% CI, 1.1, 15; likelihood ratio test P = .03). These results show that HLA-C functions as a transplantation antigen and that HLA-A and HLA-B allele mismatches are biologically important. Molecular-based methods for pretransplant assessment of class I compatibility should be implemented for the selection of unrelated marrow donors. © 1997 by The American Society of Hematology.

The class I region of the human major histocompatibility complex contains at least six genes. HLA-A and HLA-B antigens encoded by highly polymorphic genes in this region have been defined as major histocompatibility determinants in marrow transplantation. The HLA-C locus was first described 25 years ago when it became apparent that a class I gene product other than HLA-A and HLA-B could induce an alloantibody immune response. Subsequent in vitro studies demonstrated that HLA-C antigens can be recognized by cytotoxic T lymphocytes and like other class I gene products, HLA-C molecules can present peptides to T cells. HLA-C molecules also interact with class I receptors expressed on natural killer cells and regulate natural killer-mediated lysis of target cells. Although these studies demonstrate that HLA-C can participate in the immune response, the biologic role of HLA-C antigens in clinical transplantation is unknown.

Graft failure occurs in 4% of patients following unrelated donor transplantation with unmanipulated (T-cell replete) marrow. The risk of graft failure is increased with the presence of serological mismatching for HLA-A, HLA-B, or allele mismatching for HLA-DRB1. The intensity of the conditioning regimen, the use of certain pre and post-transplant immunosuppressive agents, low marrow cell dose, depletion of T cells from the marrow product, and a positive pretransplant cytotoxic cross-match. To decrease the risk of graft failure, pretransplant immunogenetic evaluation of the donor has included matching for HLA-A and HLA-B antigens, and HLA-DRB1 alleles. It has been assumed that matching for HLA-A, HLA-B, and HLA-DR might diminish any need to match for HLA-C because certain HLA-C alleles are strongly associated with specific HLA-A, B, DRB1 haplotypes, a phenomenon known as “linkage disequilibrium.” Typing for HLA-C has been limited by the lack of serological reagents capable of defining all HLA-C antigens, and hence serology has not been sufficiently reliable for clinical use.

We previously observed an unexpectedly high frequency of HLA-C mismatching in patients who experienced graft failure following unrelated marrow transplantation. To test the hypothesis that HLA-C disparity is a risk factor for graft failure, we undertook a formal retrospective analysis and employed sequencing methods and a matched case-control design to assess the contribution of HLA-C allele disparity to graft failure. Using this approach, the degree of donor-recipient compatibility at HLA-C was definitively assessed and potential confounding variables were controlled. We provide the first evidence that HLA-C functions as a transplantation antigen.

Materials and Methods

Case identification. Between May 1985 and October 1994, 556 patients with hematologic disorders and a negative pretransplant cytotoxic cross-match were treated at our center with myeloablative regimens of cyclophosphamide and total body irradiation followed by transplantation with unmodified marrow from HLA-A, B, DR matched or partially matched unrelated donors and received methotrexate and cyclosporine for prophylaxis against graft-versus-host disease. Of these patients, 521 survived for a minimum of 28 days and were evaluable for engraftment. We identified 21 patients who had graft failure according to the following definition: (1) the absolute neutrophil count did not surpass 500/μL at any time before relapse, second transplant, or death (n = 6); (2) the absolute neutrophil count decreased to less than 100/μL for at least 3 consecutive determinations at least 1 day apart after initial engraftment and did not recover before relapse, second transplant, or death (n = 6), or (3) assessment of variable number tandem repeat polymorphisms (n = 5) or in situ hybridization demonstrated the absence

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of donor T cells. The 21 graft failure cases included 18 patients with chronic myeloid leukemia (CML) (6% of 312 patients with CML), 1 with juvenile CML (20% of 5 patients with juvenile CML), 1 with acute myeloid leukemia (0.7% of 152 acute leukemia patients), and 1 with aplastic anemia (6% of 17 patients with aplastic anemia).

Selection of controls. The pool of eligible control candidates (n = 296) was restricted to patients with CML, acute myeloid leukemia, or aplastic anemia transplanted between January 1988 and October 1994 who had relapse-free survival for at least 50 days after transplant. For each graft failure case, two controls were selected who were most similar to the case with respect to the following factors (in order of decreasing importance): presence or absence of an HLA-A or HLA-B antigen disparity or HLA-DRB1 allele disparity; diagnosis; stage of disease; total body irradiation exposure and fractionation schedule; pretransplant panel reactive antibody score; year of transplant; patient/donor gender mismatch; patient age; and marrow cell dose.

Histocompatibility studies. Before transplantation, donor and recipient HLA-A and HLA-B antigens were typed by using the standard two-stage complement-dependent microcytotoxicity assay, and HLA-DRB1 and HLA-DQB1 alleles were typed by using molecular techniques.23 Donors with a single mismatch for a cross-reactive HLA-A or HLA-B antigen or a single HLA-DRB1 allele were accepted for patients younger than 36 years of age if an HLA-A, B, or DRB1 matched donor could not be identified. HLA-C antigens were typed by using standard serological techniques27 after the unrelated donor was selected according to criteria described above.

In the retrospective analysis after transplantation, alleles were assigned by amplification and sequencing of exons 2 (α1 domain) and 3 (α2 domain) of HLA-A, HLA-B, and HLA-C genes. This method identifies all alleles recognized by the World Health Organization Nomenclature Committee28 with the exception of A*0201 and A*0209, which differ only in exon 4 (α3 domain). For purposes of this study, we assumed that α1 and α2 polymorphisms provide the most relevant antigen determinants of class I molecules,29 and pairs encoding A*0201/A*0209 were therefore considered to be matched. Locus-specific amplification of HLA-A genes was performed with 5′ primer IN1CONS (TCGGGCAGGGTGTCAGCCA) and 3′ primer 18C-182T (GTGGGCCCTCTGTACCCG). Four 5′ primers were paired with the 3′ primer 18C-182T to amplify specific groups of HLA-A alleles: 2A1N (CACCCTCATGAGGTATTCTA) for A*29 and A*33, L53 (CACCCTCATGAGGTATTCTC) for A*0102, A*24, and A*30; 2A6N (CACCCTATGAGGTATTCTT) for A*0101 and A*03; and 2A8 (CACCCTATGAGGTATTCTT) for A*25, A*26, and A*66. HLA-A*11-specific amplification was achieved with 5′ primer 2A8 and 3′ primer ALI8 (TCTCTGCTGCTCCG). Amplification of HLA-B genes was performed as described.30 Exon 2 of HLA-C was amplified as described31; exon 3 was amplified with 5′ primer 66.1 (TACAAGGCCAGGCA-CAG) and 3′ primer 18CIN3.32 Amplified templates were sequenced and HLA-A, HLA-B, and HLA-C alleles were assigned by previously published methods.30,32 In addition to gene sequencing, oligonucleotide hybridization33 methods were used to characterize 15 HLA-B alleles, and primer9 methods were used to type 7 HLA-C alleles.

Statistical analysis. A matched case-control study design was employed to evaluate the contribution of HLA-C mismatching to graft failure. We constructed 21 groups of patients, each group consisting of three patients who were identical for HLA-A and HLA-B antigen and HLA-DRB1 allele match status and who were similar with regards to other risk factors for graft failure. In each group, one patient experienced graft failure and two did not. The association of HLA-C disparity with graft failure was examined by comparing the graft failure cases with their controls in regards to HLA-C mismatching.

To assess the magnitude of the HLA-C mismatch effect on graft failure, we employed conditional logistic regression models.33 This analysis allowed adjustment for imbalances within each of the 21 groups of cases and controls for HLA-A and HLA-B allele disparity, pretransplant interferon therapy (scored as ‘‘given’’ or ‘‘not given’’) and marrow cell dose.

All HLA-A and B mismatches provided both host-versus-graft (HVG) and graft-versus-host (GVH) recognition. All HLA-C mismatches with the exception of two were mismatched for HVG and GVH. Two case pairs were HLA-C mismatched for HVG only. Hence, no bias was introduced in this study by inclusion of all mismatched pairs. Since the primary endpoint of this study was graft failure, a study of GVH outcome was not undertaken.

RESULTS

Donor and recipient characteristics. Each case-control set consisted of three donor-recipient pairs: a single graft failure case pair, and two matched control pairs without graft failure. Within each of the 21 sets, the case and control pairs were identical with respect to degree of matching for HLA-A (serology), HLA-B (serology), HLA-DRB1 (allele), and to disease diagnosis (Table 1). The slight imbalance in donor-recipient gender distributions among case pairs and control pairs did not warrant correction in the analysis of graft failure. The proportion of patients who had been treated with interferon before transplantation was similar among cases (43%) and controls (33%) (P = .46). The median marrow cell dose for cases was lower than in the controls (P = .03).

HLA-A, B, C sequencing results. Serologically defined HLA-C antigens and molecularly defined HLA-C alleles were concordantly matched or mismatched by the two techniques in 52 of the 63 pairs (Table 2). In 7 of the 11 discordant pairs, a serologically defined (Cw1-Cw8) antigen was not detected, and in 4 serologically matched pairs, sequencing uncovered allele mismatching.

Among the 15 HLA-C allele mismatched graft failure case pairs, 14 were mismatched for one allele and a single pair was mismatched for both alleles. Eleven of the 16 matches involved serologically distinct allele families, Cw*01-Cw*08. Four mismatches involved Cw*1203 and Cw*1606, which cannot be detected by available antisera (HLA-C ‘‘blank’’). The remaining mismatch involved serologically indistinguishable alleles (Cw*0701, Cw*0702). Of the 14 HLA-C mismatched control pairs, 11 were mismatched for a single allele and 3 were mismatched for both alleles. Nine of these 17 mismatches involved serologically distinct allele families (Cw*01-Cw*08), 5 involved alleles corresponding to HLA-C ‘‘blank’’ antigens (Cw*1203, Cw*1505, Cw*1601), 2 involved alleles belonging to the same antigen family (Cw*0302, Cw*0303, and Cw*0701, Cw*0702), and 1 involved the Cw*0602 allele and a novel allele (unpublished). With molecular methods, at least one allele corresponding to an HLA-C ‘‘blank’’ antigen was identified in 17 (27%) of the 63 pairs.

Eighteen of the 21 case pairs and 36 of the 42 control pairs were serologically matched at HLA-A. DNA sequencing disclosed HLA-A allele mismatching in 7 of the 54 serologically matched pairs. Mismatching occurred among A*02, A*24, A*30, A*33, and A*68 alleles. Twenty case pairs and 40 control pairs were serologically matched at
HLA-B. DNA typing uncovered HLA-B allele mismatching in 16 serologically matched pairs. Mismatching occurred among B*07, B*14, B*27, B*35, B*39, and B*44 alleles. These results demonstrate that HLA-A and HLA-B alleles can differ in HLA-A, B serologically matched pairs.

**HLA-C mismatching and graft failure.** Because the design of the study controlled for HLA-A and HLA-B antigen matching and for HLA-DRB1 allele matching (Table 1), their role in graft failure was not assessed. HLA-DQB1 allele mismatching was present in two (10%) case pairs and seven (17%) control pairs and was not given further consideration in the analysis.

The degree of HLA-A, HLA-B and HLA-C allele mismatching was determined for all pairs (Table 3). Ninety percent of case pairs were mismatched for at least one locus (38% 1 locus, 43% 2 loci, 10% 3 loci) compared with 55% of control pairs (38% 1 locus, 12% 2 loci, 5% 3 loci) (Wilcoxon rank sum test P = .001). Compared with the control pairs, the case pairs had a higher incidence of mismatching for HLA-C either alone (24% v 17%) or in combination with mismatching for HLA-A and/or HLA-B (48% v 17%).

Univariable conditional logistic regression models showed that graft failure was strongly associated with HLA-C allele disparity (Table 4). Graft failure was also associated with HLA-B and HLA-A allele disparity, although the association with HLA-A allele disparity did not reach statistical significance.

Since multiple class I allele mismatches were found in a significant proportion of the cases, and since the occurrence of mismatching at these loci is not independent, a multivariable conditional logistic regression model was used to evaluate the contribution of HLA-C mismatching while controlling for any contribution of mismatching at HLA-A and/or HLA-B (Table 5). The results indicate that HLA-C mismatching is associated with an increased risk of graft failure even after accounting for the effect of mismatching at HLA-A and HLA-B. The results also suggest that disparity for HLA-A and/or HLA-B was associated with an increased risk of graft failure even after accounting for mismatching at HLA-C.

We examined multivariable models, which included covariables, to account for the potential contributions of pretransplant interferon therapy (not shown) or low marrow cell dose (Table 6) to the risk of graft failure. Estimates of the magnitude and statistical significance of the association between HLA-C mismatching and graft failure were not reduced in models that included these covariables.
HLA-C and graft failure

Table 4. Odds Ratio* of Graft Failure for an HLA-C Allele Mismatch

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>Case Pairs</th>
<th>Control Pairs</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-C</td>
<td>15 (71)</td>
<td>14 (33)</td>
<td>5.2</td>
<td>1.4, 19</td>
<td>.01</td>
</tr>
<tr>
<td>HLA-B</td>
<td>10 (48)</td>
<td>9 (21)</td>
<td>4.2</td>
<td>1.1, 16</td>
<td>.04</td>
</tr>
<tr>
<td>HLA-A</td>
<td>7 (33)</td>
<td>9 (21)</td>
<td>3.3</td>
<td>.59, 19</td>
<td>.17</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate percentage.
* Univariable conditional logistic regression model.  
† OR, odds ratio.  
‡ CI, confidence interval.

Discussion

The biological role of HLA-C in transplantation has not been previously defined, in large part because accurate methods for characterizing HLA-C gene products have been unavailable. We developed and applied molecular technology to sequence class I alleles in a retrospective matched case-control study and found that HLA-C disparity is a risk factor for graft failure following unrelated marrow transplantation. HLA-A and HLA-B sequence mismatches were also found to be risk factors for graft failure. These results demonstrate that HLA-C antigens function as transplantation determinants and that allele disparity at HLA-A and HLA-B is biologically relevant. Furthermore, molecular technology for typing major histocompatibility complex (MHC) class I genes is feasible and efforts to redirect technology should be encouraged for the selection of unrelated marrow donors.

The case-control design is a standard approach used in epidemiologic studies and was particularly appropriate in our study as it allowed HLA-C gene disparity to be investigated independently of other variables known or suspected to affect the risk of graft failure. Since the study population was restricted to patients who received unmanipulated marrow grafts and to patients with a negative cytotoxic crossmatch and since controls were selected to match cases for the presence of serological mismatching for HLA-A, HLA-B, or allele mismatching for HLA-DRB1, the intensity of the conditioning regimen, and the use of certain pre and posttransplant immunosuppressive agents, these confounding risk factors did not introduce any bias into the analysis. Adjustments were made in statistical models to account for the difference in marrow cell dose between the cases and controls. Characterization of HLA-A and HLA-B alleles in the study pairs disclosed previously undetected mismatching that could contribute to the risk of graft failure and allowed HLA-C allele disparity to be investigated independently of HLA-A and HLA-B allele disparity. Because of strong linkage disequilibrium between HLA-DRB1 and HLA-DQB1, the frequency of allele mismatching at HLA-DQB1 was not different between graft failure case pairs and control pairs. The role of HLA-DPB1 disparity in graft failure was not addressed because most unrelated transplant pairs are HLA-DPB1 mismatched.

In a previous study, disparity for serologically defined HLA-A and HLA-B antigens was found to be a risk factor for graft failure. The current study extends these findings to HLA-A and HLA-B sequence mismatches and demonstrates the functional importance of disparities that are not detectable by serologic methods. Although the design of this study did not permit evaluation of the relative contribution of mismatching at each locus, the greater prevalence of multiple class I mismatches among the graft failure cases suggests that there could be a synergistic effect of mismatching for several class I genes. The results of this analysis do not rule out the potential contribution to graft failure of other genes which may be associated with HLA-C. This hypothesis remains to be tested.

Even though marrow cell dose was included in the selection of the controls, the distribution of marrow cell dose was significantly lower in the graft failure cases than in the controls. The failure to achieve balance of marrow cell dose between the cases and controls reflects the fact that this variable was weighted below all other variables in the matching algorithm. The distribution of marrow cell dose was also significantly lower in the 21 graft failure cases when compared with the larger unrelated patient population at risk for graft failure (n = 463 evaluable patients; P < .01). The distribution of marrow cell dose among the 42 control patients in this study was similar to that of the population at risk (median, 3.3 × 10^9/kg). Since marrow cell dose was not associated with HLA-C mismatching, marrow cell dose cannot confound the relationship between graft failure and HLA-C mismatching. Indeed, the association between HLA-C disparity and graft failure risk remained significant after accounting for marrow cell dose (Table 6), indicating that HLA-C mismatching is an independent risk factor for graft failure. Because this study was not designed to specifically address the potential association between low marrow cell dose and risk of graft failure, the cumulative effect of multilocus class I disparities and marrow cell dose on the

Table 5. Odds Ratio* of Graft Failure for an HLA-C Allele Mismatch and for an HLA-A and/or B Mismatch

<table>
<thead>
<tr>
<th>Locus Mismatch</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
<th>LRT P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-C</td>
<td>4.0</td>
<td>1.1, 15</td>
<td>.04</td>
<td>.03</td>
</tr>
<tr>
<td>HLA-A and/or HLA-B</td>
<td>3.1</td>
<td>.78, 13</td>
<td>.11</td>
<td>.09</td>
</tr>
</tbody>
</table>

* Multivariable conditional logistic regression model.  
† OR, odds ratio.  
‡ CI, confidence interval.  
§ LRT P, likelihood ratio test P value.

Table 6. Odds Ratio* of Graft Failure for an HLA-C Mismatch and for Marrow Cell Dose

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-C mismatch</td>
<td>7.5</td>
<td>1.6, 34</td>
<td>.01</td>
</tr>
<tr>
<td>Marrow cell dose</td>
<td>0.4</td>
<td>.20, .90</td>
<td>.03</td>
</tr>
</tbody>
</table>

* Multivariable conditional logistic regression model. Marrow cell dose modeled as a continuous covariate (marrow cell dose/kg recipient body weight).  
† OR, odds ratio.  
‡ CI, confidence interval.  
§ LRT P, likelihood ratio test P value.
risk of graft failure remains to be examined in a larger population of graft failure cases.

At least two mechanisms involving the recognition of donor histocompatibility differences could lead to marrow graft failure.\textsuperscript{37,38} Cytotoxic T cells of the recipient could cause graft failure by recognizing class I alloantigens expressed by donor cells.\textsuperscript{35,39,40} In support of this hypothesis, we have recovered recipient-derived cytotoxic T lymphocytes specific for a donor HLA-C antigen after graft failure in a patient who received marrow from an HLA-C mismatched unrelated donor (unpublished data, April 1996). Experiments with animal models have shown that natural killer cells of the recipient can also cause marrow graft rejection.\textsuperscript{41-43} Lysis by alloreactive cytotoxic T lymphocytes requires the presence of an antigen on target cells, whereas lysis by natural killer cells occurs when specific class I epitopes are absent on target cells.\textsuperscript{8} The regulation of natural killer-mediated cytotoxicity can involve the recognition of epitopes encoded by HLA-A and HLA-B, as well as HLA-C.\textsuperscript{8,44} The underlying mechanisms of graft failure in HLA-C mismatched recipients remain to be elucidated.

The preponderance of patients with CML and the paucity of patients with acute leukemia in the graft failure group was noteworthy. CML is conventionally treated with low intensity chemotherapy as compared with the agents given to induce remission in patients with acute leukemia. As a result of having a more normal immune system before transplantation, patients with CML may have larger numbers of immunocompetent cells, which survive the conditioning regimen, thereby increasing the risk of rejection. Patients with CML might have other abnormalities that could affect the hematopoietic microenvironment in a way that impedes sustained engraftment in certain patients.

The clinical implications of this study could affect donor selection and the treatment of patients with CML who face the possibility of transplantation from HLA-C mismatched unrelated donors. Pretransplant HLA-C allele typing should be performed, and matched donors should be selected in preference to mismatched donors if possible, especially if mismatching for HLA-A and HLA-B alleles is present. If identification of a matched donor is not feasible, then it might be desirable to cryopreserve autologous stem cells in the event of graft failure. Definition of the potential synergistic effect of multiple class I allele mismatches on the risk of graft failure will make it possible to assign priorities for donor selection and would permit evaluation of new approaches for pre and posttransplant immunosuppressive therapy.

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