Cytokine Gene Polymorphisms Associating With Severe Acute Graft-Versus-Host Disease in HLA-Identical Sibling Transplants

By Peter G. Middleton, Penelope R.A. Taylor, Graham Jackson, Stephen J. Proctor, and Anne M. Dickinson

It is now well known that the initial phase of graft-versus-host disease (GVHD) involves cytokine release during preconditioning of the recipient of an allogeneic bone marrow transplant (BMT). Tumor necrosis factor alpha (TNFα), in particular, has been implicated in pathological damage and is released pretransplant due to irradiation and cytokotoxic preconditioning regimens. Interleukin-10 (IL-10), a natural immunosuppressant of TNFα, may be involved in downregulation of these responses, which may be an individual patient-specific effect. In this study, we determined the genotype for polymorphisms associated with TNFα and IL-10 in 80 potential allo-BMT recipients and correlated the genotype with the severity of GVHD in 49 patients for whom clinical data relating to GVHD was available. The widely studied TNFα -308 polymorphism does not show any significant associations, but the d3 homozygous allele of the TNFα microsatellite is preferentially associated with grade III/IV GVHD (7 of 11 patients) compared with its occurrence in 8 of 38 patients with grade 0/II GVHD (P = .006). Alleles of the IL-10 -1064 promoter region microsatellite polymorphism that possess greater numbers of dinucleotide (CA) repeats also significantly associate with more severe GVHD. This region has been demonstrated to be important in the regulation of the IL-10 promoter. Eighteen of 38 patients with grade 0-II GVHD possessed alleles with greater numbers (12 or more) of dinucleotide repeats, compared with 9 of 11 cases with grade III-IV GVHD (P < .02). Of the 38 patients with grade 0-II GVHD, 3 of 38 had a both TNFα/d3 and IL-10 (12-15) genotype, compared with 6 of 11 patients with grade III-IV GVHD (P < .001). There was no association of either the TNFα or IL-10 microsatellite polymorphisms with mortality (P = .43 and .51, respectively). Our results suggest that patient cytokine gene polymorphism genotypes may influence GVHD outcome by affecting cytokine activation during the pretransplant conditioning regimens, and these results are the first to suggest a genetic predisposition to this important transplant-related complication.

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produced high levels of TNFα and low levels of IL-10.15,16 A number of polymorphisms of the IL-10 gene have been described, including single base (G/A) polymorphism (−1082) associated with altered levels of in vitro IL-10 expression16 and microsatellite polymorphism (−1064) mapping near candidate IL-10 gene control elements.17 Recent findings suggest a role for some of these polymorphisms in determining rejection in heart transplant recipients.15,16

In this present study we have determined the BMT recipient TNFα and IL-10 genotype for gene polymorphisms associated with either reported cytokine productivity or candidate gene control regions and correlated results with HLA phenotype and clinical outcome, including the incidence and severity of posttransplant GVHD.

MATERIALS AND METHODS

Patients and normal controls for polymorphism studies. Eighty patients being considered for transplant with underlying hematological malignancies predominantly acute leukemia (ALL) or chronic granulocytic leukemia (CGL) were tested for TNFα and IL-10 polymorphism allele frequencies and genotype. Twenty-eight normals (laboratory personnel) were also tested for TNFα and IL-10 polymorphism allele frequencies and genotype. The results are included in Fig 1A and B.

Cytokine gene polymorphism studies were performed on all patients, including the 49 of 80 patients who subsequently underwent BMT from an HLA- and HLA-DR–matched, mixed lymphocyte culture-negative, sibling donor. The TNFα and IL-10 polymorphism results from these patients were correlated with incidence and severity of GVHD. These patients included 32 males and 17 females (median age, 33; age range, 14 to 47 years). All 49 patients received non–T-cell–depleted marrow grafts. The underlying diseases were CGL (n = 12), acute myeloid leukemia (AML; n = 18), ALL (n = 15), Hodgkin’s disease (HD; n = 1), non-Hodgkin’s lymphoma (NHL; n = 1), and myelodysplastic syndrome (MDS; n = 2).

Conditioning regimens and GVHD prophylaxis. Before January 1991, standard conditioning for the patients with acute leukemia was with cyclophosphamide (120 mg/kg) and total body irradiation (TBI; 12 Gy in 6 fractions in 3 days at 25 cGy/min). Since January 1991, the TBI dose has remained the same, but melphalan (3 mg/kg) has been substituted for the cyclophosphamide. Patients with MDS were conditioned with cyclophosphamide alone. Patients with CGL were usually conditioned with busulphan (4 mg/kg/d for 4 days) and cyclophosphamide.

The majority of patients were treated with either cyclophosphamide or melphalan plus TBI (Table 1). The number of patients treated with these two types of conditioning regimes were equally distributed between the cohorts of patients developing GVHD grades 0-II and GVHD III-IV (30 of 38 and 9 of 11, respectively; χ² = .043; df = 1; P = .853; Tables 1 and 2).

GVHD prophylaxis and therapy. GVHD prophylaxis was with cyclosporin alone, administered intravenously at a dose of 5 mg/kg until oral treatment could be tolerated, when the same dose was administered by mouth. Once clinical GVHD was diagnosed, standard therapy was administered with high-dose steroids; if there was no immediate response, this was followed by increased cyclosporin (5 to 10 mg/kg).

Ethics committee approval and informed consent. This study was approved by the local ethics committee and informed consent was obtained from all patients and normal controls under study.

HLA typing. All patients in this study were tested by conventional serology for HLA A and B alleles along with low resolution molecular typing of DRB1 using polymerase chain reaction (PCR) sequence-specific primer.18 All tissue typing was routinely performed at the Northern Blood Transfusion Service (Newcastle, UK).

TNFα and IL-10 genotypes. Patient genotypes were determined for the TNFα −308, TNFα microsatellite, and IL-10 −1064 microsatellite polymorphisms essentially as described.5,10,19 PCR products were separated by gel electrophoresis on polyacrylamide gels (10%, 19:1 acrylamide:bis) and visualized by silver staining. All PCR products were
compared with a control of known genotype (TNF-308 1/2, TNFd1/d3, IL-10 i9/13) to ensure accurate interpretation. Allele designations, eg, d1/d3, 9/13 etc, are as described in the original reports of these polymorphisms.7,10,19

Statistical analyses. Comparisons between patient groups were made using the x² test for heterogeneity.

RESULTS

Incidence and severity of GVHD. GVHD was diagnosed and graded according to previously published criteria.20 Of the 49 patients transplanted, 9 showed no evidence of acute GVHD. Fourteen patients developed grade I GVHD: 10 with skin alone, 3 with skin plus gastrointestinal involvement, and 1 with gastrointestinal involvement alone. Fifteen patients developed grade II GVHD; all had skin involvement, 4 had hepatic involvement (2 with gastrointestinal disease), and 3 had skin and gastrointestinal involvement with no evidence of liver disease. Eleven of 49 patients developed severe (grade III-IV) GVHD. All 9 patients with grade III disease had skin and gastrointestinal GVHD, together with liver involvement in 4 patients. In this whole cohort, only 2 patients died of GVHD (Tables 1 and 2).

TNFα-308 polymorphism. Of the 80 patients tested, 3 were homozygous for the uncommon TNF2 allele (the allele frequencies would predict 2.1 homozygotes) and 20 cases were heterozygous (TNF1/2). The allele frequencies of TNF1 and TNF2, respectively, were 0.838 and 0.162. These results were
comparable with those quoted for a normal Caucasian population (0.84 and 0.16, respectively, measured on 40 individuals [80 alleles]). In common with other studies, a strong association (0.84 and 0.16, respectively, measured on 40 individuals comparable with those quoted for a normal Caucasian population; 23.1 \pm 15; 19/13 predicted v observed; 12.75 \pm 13).

**Allele frequencies of the TNF\(_d\) and IL-10 microsatellite polymorphisms.** The allele frequencies of TNF\(_d\) and IL-10 microsatellites found in the 80 patients tested, along with those previously reported in other studies, and a panel of 28 unselected normals are shown in Fig 1A and B. The observed allele frequencies were similar to those reported elsewhere, indicating that the appropriate allele designations were made for patient and normal control cohorts. The frequency of predicted homozygotes (as predicted by Hardy-Weinberg equilibrium) for the more common alleles compared with the observed frequencies, suggesting that homozygous genotypes were being appropriately assigned in the study group (TNF\(_d\)/d3/d3 predicted v observed; 23.1 v 26; IL-10 i9/i9 predicted v observed; 14.45 v 15; i9/i3 predicted v observed; 12.75 v 13).

**TNF\(_d\) and IL-10 microsatellite polymorphisms and association with GVHD.** The d3 allele of the TNF\(_d\) microsatellite was preferentially associated with grade III/IV GVHD, with 7 of 11 patients possessing the d3/d3 genotype, compared with its occurrence in 8 of 38 patients with grade 0-II (\(\chi^2 = 7.598; df = 1; P < .006;\) Tables 1 and 2).

Alleles of the IL-10 \(-1064\) promoter region microsatellite polymorphism that possessed greater numbers of dinucleotide repeats (alleles 12, 13, 14, or 15, as described by Eskdale and Gallagher\(^{15}\)) also significantly associated with more severe GVHD. Eighteen of 38 patients with grades 0-II GVHD possessed high repeat number alleles (12-15; Table 1), compared with 9 of 11 patients with grades III-IV GVHD only (\(\chi^2 = 5.443; df = 1; P < .02;\) Table 2).

If both TNF\(_d\) and IL-10 genotypes were considered together, there appears to be a retrospective association with GVHD severity and occurrence. Of the 38 patients with grade 0-II GVHD, 3 of 38 had a TNF\(_d\)/d3/d3/IL-10 (12-15) genotype, compared with 6 of 11 patients with grade III-IV GVHD (\(\chi^2 = 14.11; df = 1; P < .001;\) Table 2).

**Table 2. Patient Characteristics and Cytokine Genotypes With the Development of GVHD Grades III-IV**

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>CR</th>
<th>HLA Type</th>
<th>TNF –308 Genotype</th>
<th>TNF(_d) Genotype</th>
<th>GVHD Occurrence</th>
<th>Survival</th>
<th>Posttransplant (mo)</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>CGL</td>
<td>Cy/TBI</td>
<td>A2 A9 (24) B7 Bw22 (55) Cw3</td>
<td>F1/F1</td>
<td>i3/i3*</td>
<td>d3/d3*</td>
<td>III</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>AML</td>
<td>M/TBI</td>
<td>A2 A30 (19) B13 B9 (16) DR17 DR8</td>
<td>F1/F1</td>
<td>i9/i3*</td>
<td>d3/d3*</td>
<td>II</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>ALL</td>
<td>M/TBI</td>
<td>A28 A30 (19) B27 B44 (12) DR1501-1503</td>
<td>F1/F1</td>
<td>i11/i3*</td>
<td>d3/d3*</td>
<td>II</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>CGL</td>
<td>BuMu</td>
<td>A1 A11 B5 B57 (17) DR4 DR71 (3)</td>
<td>F1/F1</td>
<td>i0/12/12*</td>
<td>d4/d4</td>
<td>III</td>
<td>2</td>
<td>Fungal infection</td>
</tr>
<tr>
<td>43</td>
<td>AML</td>
<td>M/TBI</td>
<td>A25 A31 B18 B40 DR2 DR4</td>
<td>F1/F1</td>
<td>i8/15*</td>
<td>d3/d3*</td>
<td>III</td>
<td>1</td>
<td>Allotoxicity</td>
</tr>
<tr>
<td>44</td>
<td>CGL</td>
<td>Cy/TBI</td>
<td>A2 A3 B8 B5 DR3 DR5</td>
<td>F1/F2</td>
<td>i8/13*</td>
<td>d3/d3</td>
<td>III</td>
<td>2</td>
<td>GVHD and viral infection</td>
</tr>
<tr>
<td>45</td>
<td>AML</td>
<td>M/TBI</td>
<td>A2 A3 B7 DR2 DR4</td>
<td>F1/F1</td>
<td>9/14*</td>
<td>d3/d3</td>
<td>III</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>ALL</td>
<td>M/TBI</td>
<td>A1 A3 B17 B15 DR(_d) DR7</td>
<td>F1/F1</td>
<td>i9/i9</td>
<td>d4/d4</td>
<td>III</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>AML</td>
<td>M/TBI</td>
<td>A1 A3 B8 B35 DR1 DR17 (3)</td>
<td>F1/F2</td>
<td>i9/i3*</td>
<td>d1/d4</td>
<td>III</td>
<td>122</td>
<td>Nontransplant?? related death</td>
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<tr>
<td>48</td>
<td>MDS</td>
<td>BuCy</td>
<td>A11 A32 B7 B4 DR4 DR7</td>
<td>F1/F1</td>
<td>i9/i9</td>
<td>d3/d3</td>
<td>IV</td>
<td>2</td>
<td>GVHD</td>
</tr>
<tr>
<td>49</td>
<td>AML</td>
<td>M/TBI</td>
<td>A2 A29 (19) B44 (12) DR4 DR7</td>
<td>F1/F1</td>
<td>i10/i13*</td>
<td>d3/d3*</td>
<td>IV</td>
<td>2</td>
<td>VOD</td>
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</tbody>
</table>

Characteristics and results on patients with grade III-IV GVHD showing unique patient number (UPN), diagnosis, conditioning regimes available HLA typing results, genotype for the TNF –308, IL-10 –1064, and TNF\(_d\) microsatellite polymorphisms, occurrence of acute GVHD, survival, and cause of death.

Abbreviations: VOD, veno-occlusive disease; Cy/TBI, cyclophosphamide/TBI; M/TBI, melphalan/TBI. C/Bu, Busulphan; M/VP16/B, melphalan/ataposide/BCNU.

*Patients who possessed an IL-10 microsatellite allele with greater numbers of dinucleotide repeats (alleles 12-15).
†Patients who were homozygous for the TNF\(_d\) allele (d3/d3).
In this present study, the d3 allele of the TNFd polymorphism was found to be associated with severe GVHD grades III/IV but not with overall clinical survival. This result suggests that the effect seen in the d3/d3 TNFd genotype may be susceptible to immunosuppression as suggested by Turner et al. in studies on heart transplant recipients in which the effect was related to the degree of immunosuppression. The increased incidence of GVHD in the homozygous d3/d3 cohort was associated with increased GVHD, but also this subpopulation was possibly more responsive to the GVHD therapy in the form of increased cyclosporin and methotrexate and therefore did not succumb to fatal GVHD. A similar effect may be the explanation for the lack of correlation with GVHD mortality and IL-10 (12-15) allele polymorphisms. This hypothesis can only be tested in larger cohorts of patients with and without increased GVHD prophylaxis and altered therapy. Our studies have also shown that the −308 polymorphism was not associated with GVHD severity.

A large number of studies have looked unsuccessfully for associations between TNF2 genotype and disease incidence, severity, or susceptibility in a range of immunoregulatory disorders. These include studies on rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, lichen sclerosus, and ankylosing spondylitis. An association between the TNF2 allele and the incidence of insulin-dependent diabetes mellitus has been reported, but this association is not independent of the HLA type and probably reflects linkage disequilibrium between the TNF2 polymorphism and the ancestral HLA haplotype. A similar association has been seen in rheumatoid arthritis patients with systemic lupus erythematosus. An association between disease incidence and the TNF2 allele that is independent of the HLA type has been reported in studies on asthma. Taken together, these findings indicate that the TNF2 genotype may not be important in immunoregulatory disorders and that other candidate loci or genetic elements must be considered.

A proven association of cytokine gene polymorphisms with GVHD would undoubtedly enable the clinician to modify therapy in those patients predicted to develop GVHD by virtue of their underlying cytokine genotype. The study reported here documents the possible role of IL-10 gene polymorphism in GVHD. Analysis of both the TNFα and IL-10 polymorphisms and GVHD severity was based on knowledge that grade 0-II GVHD is more susceptible to therapeutic intervention than grades III-IV, which are often fatal. This increase in risk of severe GVHD if possessing risk-associated alleles for both the TNFα d3/d3 and IL-10 (12, 13, 14, and 15) genotype was highly significant ($P < .001$). However, possession of either genotype alone was also significant. These results suggest both an interacting role for TNFα and IL-10 and a role for both as independent indicators of severity of GVHD. This further suggests that other factors undoubtedly regulate TNFα and IL-10 production and play a role in the ultimate degree of GVHD.

The extension of these studies to include other indicators/predictors of GVHD in HLA-identical siblings, such as minor histocompatibility testing, HTLp frequency analysis, cytokine production, and/or predicting GVHD using a skin explant model, may allow the development of an individual risk index for GVHD. Such a risk index would allow for improved management for GVHD, which is still the major cause of morbidity and mortality after allogeneic BMT.

Recent approaches to therapy include engineering a shift in cytokine profiles before BMT as a way to attenuate inflammatory processes of GVHD. These approaches include the possible use of anti-TNFα antibodies and recombinant IL-10, a strong inhibitor of TNFα. The finding of 8 of 9 deaths from disease relapse in the grade 0-I GVHD group suggests that prior knowledge of a patient’s risk status for cytokine production might be of value not only in attenuating the effects of GVHD in high-risk individuals, but also in potentially upregulating the response in GVHD low-risk individuals to manipulate the graft-versus-leukemia (GVL) effect. Such management options would only become reality if accurate GVHD/GVL predictions for an individual patient could be made.

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