Immune modulation to prevent antibody-mediated rejection after allogeneic hematopoietic stem cell transplantation

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A B S T R A C T

It has been shown that antibodies to donor CD34+/VEGFR-2+ stem cells or antibodies against mismatched HLA are associated with graft rejection after hematopoietic stem cell transplantation (HSCT). CD34+/VEGFR-2+ positive stem cells have been implicated to play a major role in engraftment after HSCT. In this study we treated four patients with an imminent risk of antibody-mediated rejection with immune modulation, i.e. plasma exchange, intravenous immunoglobulin (IVIG), and rituximab before HSCT. Three of the patients had been previously transplanted and rejected their initial grafts after 12 months, 1 month, and less than 1 month, respectively. The fourth patient was not transplanted previously but had HLA directed antibodies present against the graft.

During the immune modulatory treatment we followed the pattern of antibodies in sera using FACS and microcytotoxicity assay.

We could show that two patients had antibodies against donor CD34+/VEGFR-2+ cells while the other two had antibodies directed against HLA. All four patients tolerated the immune modulatory regimen without any side effects.

In this preliminary study we show that immune modulatory treatment may be used to reduce antibody levels and to prevent rejection after HSCT. In two of the three patients which experienced previous rejections and had detectable anti-HLA or anti-CD34+/VEGFR-2+ antibodies, immune modulation resulted in engraftment. In the fourth patient with known anti-HLA-class I antibodies, the treatment also resulted in engraftment. Our results encourage further studies regarding this treatment regimen.

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1. Introduction

Graft failure after allogeneic hematopoietic stem cell transplantation (HSCT) has become an increasing problem since the introduction of reduced intensity conditioning (RIC)[1], the use of T-cell depleted marrow [2], and wider use of HLA-mismatched donors [3].

Several immunological mechanisms may lead to graft failure. Typically, this failure is due to recipient T-cells [4–8]. Whether or not antibodies can cause HSCT rejection is controversial [9–11], although recent data have demonstrated that the presence of preformed donor-reactive antibodies is a strong barrier to bone marrow engraftment in allo sensitized recipients [12–14]. In organ allograft rejection, allo antibodies contribute to both early and late graft loss [15].

Recently, studies have indicated that CD34+/VEGFR-2+ cells from adult bone marrow or cord blood may generate both hematopoietic and endothelial cells in vitro [17]. This cell population also appears to be important for engraftment after HSCT [18]. In a recent study, we showed that there is a correlation between the presence of recipient antibodies to donor CD34+/VEGFR-2+ cells and rejection [19]. Rejection of the graft was associated with a high mortality rate. In the present study, we tried to remove allo specific antibodies to avoid rejection after HSCT using immune modulation. Such immune modulation has previously been used successfully before and after renal transplantation [20].

2. Materials and methods

2.1. Patients

We included three patients with previous rejections and one patient with known anti-HLA antibodies prior to HSCT, and treated them all with plasma exchange, intravenous immunoglobulin (IVIG), and rituximab before HSCT. Two patients had antibodies to donor...
CD34+/VEGFR-2+ cells [19] and the other two had anti-HLA antibodies due to extensive blood transfusions before transplantation. Patients 1 and 2 received CsA and MTX as GVHD prophylaxis [21]. Patients 3 and 4 were given CsA and steroids [22]. Patient and donor characteristics are listed in Table 1.

2.2. Plasma exchange and rituximab

Plasma exchange was conducted with Cobe Spectra. At each session, one plasma volume was drawn from the patient and replaced with the same amount of fresh plasma. The patient received Calcium-Sandoz (9 mg/ml) as continuous infusion during the whole process as prophylaxis against side effects due to citrate.

Patients 1 and 2 underwent plasma exchange for five consecutive days while patient 3 had 10 days of plasma exchange and patient 4 had 4 days of plasma exchange prior to transplantation and 3 sessions after transplantation. Patients 1 and 2 also received rituximab (375 mg/m²) 7 days before HSC; patient 3 received rituximab (375 mg/m²) 13 days before transplantation, and patient 4 received rituximab (375 mg/m²) 15 days before HSCT. In addition, all patients received 4-5 days of treatment (day 5 or 4 to day 1) with antithymocyte globulin (ATG; Genzyme, Cambridge, MA, USA) at a total dose of 6-8 mg/kg. Patients 1 and 2 also received a single dose of Campath (10 mg) at day −6. All patients received one dose of immunoglobulin substitution after the entire series of plasma exchange sessions (0.25 g/kg).

2.3. Chimerism analysis and definition of rejection

For chimerism analysis, peripheral blood (PB) samples were collected from the donor and recipient before transplant and from the recipient weekly up to 3 months after HSCT and monthly thereafter. DNA from donor and recipient pre-transplantation samples was extracted using standard protocols (MagnNA Pure; Roche, Basel, Switzerland). To evaluate lineage-specific chimerism, CD3+, CD19-, and CD33-positive cells were selected using immune magnetic beads (Dynal, Oslo, Norway). The methodology and sensitivity of chimerism analysis is described elsewhere [23]. Rejection is defined as either no detection of donor cells after HSCT or complete loss of donor cells after initial engraftment. In all patients with rejection, relapse of the underlying disease was excluded either by morphological examination of bone marrow aspirates or by RT-PCR of BCR-ABL or other relevant chromosomal aberrations.

2.4. Serum samples

Patient serum was separated from whole blood by centrifugation and stored at −20 °C until use. Pre-transplant sera were obtained immediately before transplantation. Post-transplant sera were obtained on a weekly basis.

2.5. Detection of panel-reactive antibodies

Detection of panel-reactive antibodies using flow cytometry was performed as previously described [24]. Briefly, 20 μl of serum from the patient was incubated for 30 min at 22 °C with 2.5 μl of HLA class I or II antigen-coated beads (One Lambda, Canoga Park, CA, USA). Following incubation, the beads were washed and resuspended in 250 μl PBS containing 0.5% formaldehyde. Detection of possible bound panel-reactive antibodies was performed using the flow cytometer FACSCalibur from Becton Dickinson (BD Biosciences, Sweden) and the samples were analyzed using CELLQuest software (BD Biosciences). Samples expressing <4.1% reactivity for class I and <2.9% for class II were considered negative. To estimate the effect of the plasmapheresis, undiluted sera as well as serum diluted 2-, 4-, and 16-fold were subjected to PRA determination.

2.6. Isolation of CD34+/VEGFR-2+ cells

At the time of transplantation, donor bone-marrow cells or granulocyte colony stimulating factor (CSF)-mobilized peripheral blood cells were isolated and cryopreserved in liquid nitrogen. Donor peripheral blood mononuclear cells (PBMCs) expressing VEGFR-2 and CD34 were isolated using antibodies to the specific molecules (Dynal, Oslo, Norway). The methodology and sensitivity of chimerism analysis is described elsewhere [23]. The positive fraction (CD34+/VEGFR-2+) was used for the engraftment.

Table 1

<table>
<thead>
<tr>
<th>Diag.</th>
<th>Sex (D/R)</th>
<th>Age</th>
<th>Donor</th>
<th>Conditioning</th>
<th>GVHD prophylaxis</th>
<th>Donor Age</th>
<th>S.C.-source + dose CD34+ x10^6/kg</th>
<th>Blood-group (D/R)</th>
<th>HLA-match (A,B,C,DR,DQ)</th>
<th>Time to rejection (months)</th>
<th>Transf. pre-HSCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pat. 1</td>
<td>M/F</td>
<td>1</td>
<td>UD</td>
<td>MAC 1)</td>
<td>CsA + MTX</td>
<td>25</td>
<td>BM18.4</td>
<td>0+/-0/+</td>
<td>10/10</td>
<td>12</td>
<td>Yes</td>
</tr>
<tr>
<td>CML F/F</td>
<td>12</td>
<td>UD</td>
<td>RIC 2</td>
<td>CsA + MTX</td>
<td>34</td>
<td>BM2.5</td>
<td>9/10</td>
<td>11/12</td>
<td>&lt;1</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>F/F</td>
<td>UD#2</td>
<td>RIC 4</td>
<td>CsA + MTX</td>
<td>27</td>
<td>BM3.6</td>
<td>0+/-0/+</td>
<td>9/10</td>
<td>1</td>
<td>&lt;1</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Pat. 3</td>
<td>7/M</td>
<td>11</td>
<td>RIC 6</td>
<td>CsA + Steroids</td>
<td>CB TNC: 7.9x10^7</td>
<td>A+/A+</td>
<td>5/6</td>
<td>&lt;1</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fanconi</td>
<td>7/M</td>
<td>53</td>
<td>MAC 8</td>
<td>CsA + Steroids</td>
<td>CBx2 CB1/TNC:3.4x10^7</td>
<td>A+/B+/A+</td>
<td>CB1/TNC:1.3x10^7</td>
<td>4/6</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1) Etoposide 300 mg/m² ×1, Bu 1 mg/kg ×4 for 4 days, Cy 60 mg/kg ×1 for 2 days, ATG 8 mg/kg for 5 days; 2) Flu 30 mg/m² for 4 days, Cy 60 mg/kg ×4 for 2 days, ATG 8 mg/kg ×4 days; 3) Flu 30 mg/m² for 6 days, Bu 1 mg/kg ×4 for 2 days, ATG 8 mg/kg ×4 days; 4) TLI 2 Gy for 3 days, Flu 30 mg/m² for 5 days, Melphalan 140 mg/m² (2) ×1, ATG 6 mg/kg for 4 days; 5) Flu 30 mg/m² for 5 days, Holoxan 3 g ×1 for 2 days, Cy TBI for 5 days, ATG 6 mg/kg ×2 days; 6) Flu 30 mg/m² for 5 days, Cy TBI for 5 days, ATG 6 mg/kg ×4 days; 7) Flu 30 mg/m² for 4 days, Cy TBI ×1, Bu 1 mg/kg ×4 for 4 days, Cy 60 mg/kg ×2 days, ATG 6 mg/kg ×4 days.

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2.7 Flow cytometric assay for detection of donor-specific antibodies to CD34+/VEGFR-2+ cells

For the assay, $5 \times 10^5$ CD34+/VEGFR-2+ enriched donor cells were incubated with 50 µl of patient serum for 30 min at RT, and then washed with PBS. Ten microliters of 1:10 diluted fluoresceinated F(ab')2 fragments of goat anti-human IgG (Fc-specific) antibodies or IgM (both from Jackson Immuno Research, West Grove, PA, USA) were added and incubated in the dark for 30 min. Heat-inactivated serum from a non-immunized male with blood group AB served as a negative control. The cells were then analyzed on a flow-cytometer (FACSorter; Becton Dickinson). A shift in the mean fluorescence of 20 channels in the test sample as compared to the negative control was considered positive, and determined as previously published [15]. The details of the lysis scale are given in Fig. 2 and Table 2.

2.8 Microcytotoxicity assay

To study the functional capacity of antibodies specific to donor CD34+/VEGFR-2+ cells, we tested the in vitro ability of these antibodies to fix complement. For this purpose, we used the microcytotoxicity assay as described earlier [15]. Reactions were considered positive when there was lysis of more than 10% above background as compared to the negative control.

3. Results

3.1. Patient outcome

Patient 1, a 1-year-old girl with hemophagocytic lymphohistiocytosis (HLH), received an HLA-A,-B,-C, DRB1,-DQ, and DP allele-matched bone marrow from an unrelated donor with the same ABO blood group. Before the first transplantation, she was given myeloablative conditioning (Table 1).

Chimerism analysis at 2 months after HSCT showed increasing levels of recipient cells in CD3, CD19, and CD33 cell lineages and she was therefore given four escalating doses of donor lymphocyte infusions (DLIs): $0.7 \times 10^7$, $2 \times 10^7$, $5 \times 10^7$, and $2 \times 10^8$ cells/kg. In spite of DLI treatment, the graft was rejected 1 year after HSCT. Since a small amount of donor T-cells was detected in peripheral blood and no other stem cell donor was available, it was decided to perform a second HSCT with the same donor. Since antibodies to donor CD34+/VEGFR-2+ cells were detected the patient was treated with rituximab, plasma exchange, and IVIG before receiving RIC and a second HSCT (Table 1). Since antibody-mediated rejection was suspected in both the first and second transplant, a threatening rejection caused by antibodies against donor CD34+/VEGFR-2+ cells was anticipated. She was treated with rituximab, plasma exchange, and IVIG before receiving RIC and a third HSCT. She was diagnosed with grade II acute GVHD, and prednisolone treatment (2 mg/kg) was instituted (Fig. 1). Chimerism analysis 2 months after HSCT showed almost complete donor chimerism (~95%) in all cell lineages (Fig. 1).

Two months after she was discharged, a bone marrow aspiration was performed, which showed the development of a CMML blast crisis. Twelve months after her third transplantation, she is now treated with hydroxyurea and suffers from severe chronic GVHD of the skin.

Patient 2, a 13-year-old girl with Fanconi anemia, was first grafted with cord blood (CB) with a DRB1 antigen mismatch, but had matched blood groups. The patient received RIC (Table 1). Chimerism analysis after 3 weeks showed only recipient cells in all three lymphoid cell lineages. The patient was still pancytopenic.

Before the first transplantation, HLA class I and II antibodies were detected with Flow PRA. Two units of CB were used for re-transplantation to increase the cell dose and to minimize the risk of antibody-mediated rejection. Plasma exchange was performed for 10 days before re-transplantation to reduce anti-HLA antibodies. Antibody levels were analyzed with Flow PRA after every plasma exchange. The patient was also given mesenchymal stem cells (MSCs) together with double CB to modulate recipient and donor immunity as was earlier shown to be possible in a haplo-identical setting [24]. Three weeks after the second transplantation, patient 2 was still pancytopenic. In addition, chimerism analysis showed only recipient cells. The patient died 5 weeks after the second HSCT due to haemorrhage.

Patient 4, a 53-year-old male, underwent HSCT due to secondary acute myeloid leukemia (AML) after previous Hodgkin lymphoma. Prior to CB transplantation panel-reactive antibodies (PRAs) against HLA class I were detected using flow cytometry. The patient had high antibody levels to several HLA-B antigens, but the specific HLA-B antigen mismatch between the patients and CB unit 1 could not be analyzed specifically. It was therefore decided to add another unit of CB (unit 2) with HLA B 6/6 match and negative Flow-PRA, but with a low cell dose of only 1.3 $\times 10^7$ total nucleated cells (TNC) per kg.

After experience from patient 3 without monitoring of antibody levels after HSCT and with an antibody rebound effect retrospectively confirmed, we decided to change the treatment for patient 4. Plasma exchange was therefore performed 4 times prior to transplantation and 3 times after HSCT. The first chimerism on day +23 showed 100% donor engraftment of CB unit 1. However, the patient did not reach ANC (a neutrophil count) $>0.5 \times 10^9$ until day +48.

3.2. Reactivity of patients’ antibodies to donor CD34+/VEGFR-2+ cells and their association with rejection and cytotoxicity

FACS and microcytotoxicity assays against donor CD34+/VEGFR-2+ cells were performed with sera from patients obtained before and after HSCT. Using FACS, patient 1 had antibodies that were reactive to donor CD34+/VEGFR-2+ cells. These antibodies were seen shortly after the first transplantation and at the time of rejection. In the microcytotoxicity assay, these antibodies caused cell lysis of donor CD34+/VEGFR-2+ cells. Serum taken at the time of rejection and just before plasma exchange showed complete lysis, in contrast to serum taken shortly after plasma exchange where no lysis was seen. Serum taken after HSCT again showed cell lysis, most probably due to a rebound effect of antibodies (Fig. 2).

In patient 2, by FACS, antibodies to donor CD34+/VEGFR-2+ cells were detected before and after the first and second transplantation, but not before the third transplant. However, these results were obtained after the third transplant. Since the patient had rejected the two previous grafts probably due to antibodies to CD34+/VEGFR-2+ cells, she received immune modulatory treatment before the third transplant. The serum of this patient was toxic to the CD34+/VEGFR-2+ cells of the first and second donors both before HSCT and at the time of rejection. However, with the third donor no lysis of donor CD34+/VEGFR-2+ cells was seen and this graft was accepted (Table 2).

3.3. Detection of panel-reactive anti-HLA class I and II antibodies in patient sera

Before his second HSCT, patient 3 was treated with repeated plasma exchanges. As shown in Fig. 3A-B, PRA values increased following plasma exchange treatment in both undiluted and diluted sera from the patients. However, a rebound effect was seen 26 days after the last plasma exchange, which may explain why the patient never engrafted. Unfortunately, the analysis of samples after HSCT using PRA determination was conducted too late and no plasma exchange after HSCT was administered.
4. Discussion

Unlike solid organ transplantation, the role of antibodies in HSCT rejections has been debated [9,11]. Recently, two studies demonstrated that humoral immunity may be a major barrier to allogeneic bone marrow engraftment in allosensitized recipients [13,14]. These studies suggest that humoral mechanisms may contribute to graft rejection after HSCT.

We have previously shown that antibodies to donor CD34+/VEGFR-2+ cells are associated with rejection after HSCT [19]. Since there is a correlation between rejection and high morbidity and mortality, our aim in this study was to find a way of removing allo-antibodies before and after HSCT. In organ transplantation there are now several ways of removing antibodies that may cause graft failure [16,25,26].

We used a similar protocol to that used by Tyden et al. in patients with antibodies to donor CD34+/VEGFR-2+ cells before and after plasma exchange, transplantation, and GVHD in patient 1 associated with the re-transplantation. Positive control serum = 8, Negative control serum = 0. Microcytotoxicity assay scale (Mca-scale): 0 = 0–15%, 2 = 15–25%, 4 = 25–50%, 6 = 50–75%, 8 = 75–100% dead cells.

Fig. 1. Result of chimerism analysis over time in patients 1, 2, 3 and 4 showing percent recipient CD3+ and CD33+ cells above and below the line respectively.

In patient 4, we changed the treatment as mentioned earlier, and plasma exchange was also performed after HSCT. This resulted in significant and sustained reduction of HLA class I antibodies (Fig. 3C).

antibodies [16]. We treated them with rituximab, plasma exchange, and one dose of IVIG before transplantation to avoid humoral rejection. While the treatment modality is not yet part of standardized hospital care, modifications were done chronologically on patient to patient basis based on previous experience.

Patient 1 was not treated with further plasma exchange due to her poor clinical condition. She developed acute GVHD grade III and received standard GVHD treatment. Because of the poor response, she was also given MSCs as described earlier [27]. After the development of severe acute GVHD, she became a complete donor chimera in all three cell lineages. Mixed chimerism does not protect against GVHD; however, after development of GVHD, patients may show complete donor chimerism as described earlier [28,29]. Alloreactive T-cells and GVHD most probably eliminated recipient hematopoiesis including B-cells, and prevented a threatening rejection. However it should be taken in to account that in some cases changes in the immunosuppressive regimen or immune modulatory treatments such as MSCs may also have an effect on the chimeric pattern.

In patient 2, immune modulation including plasma exchange was feasible with no side effects. However, it was unnecessary because the patient had no anti-CD34+/VEGFR2+ antibodies against this donor. Thus, in patients with such antibodies, microcytotoxicity to such cells from potential donors should be screened for in advance if possible.

Patient 2 developed an AML-like CML blast crisis shortly after the third transplantation. It could not be determined cytogenetically whether it was a novel AML or transformation of the original CML. It has been reported that rituximab may cause leukemia-relapse in patients receiving rituximab as a therapy for chronic GVHD [30].

Patient 3, who was transplanted twice with CB, had anti-HLA antibodies. Since this was detected before the second HSCT with Flow-PRA, the patient was treated with plasma exchange, rituximab, and double CB to increase the cell dose. In addition MSCs as engraftment support was tried, as this was earlier shown to have effect in a haplo-identical setting [24]. Despite these treatments, he never engrafted.

The use of rituximab to avoid humoral rejection after HSCT has previously been successfully used [31]. It is well known that patients who have received multiple blood transfusions before transplantation are more prone to reject their grafts [32,33]. One or two HLA–antigen mismatches may be acceptable [34,35]. However, engraftment is delayed and failure of grafting is higher using CB transplants than using bone marrow [36]. In a recent study by Takanashi et al., the
also performed plasma exchange before and after HSCT. This resulted in a
importance of the rebound antibody production effect and we therefore
more sessions were required[16].

transplantation, or if the titers following the last session exceeded 1/8,
rebound effect of antibody production is well known [16]. There
appears to be a correlation between rebound antibodies and antibody

authors could detect HLA directed antibodies in a reasonable high
frequency of CB transplanted patients. The presence of graft directed
antibodies was significantly correlated to less platelet and neutrophil
recovery [37].

In patients 1 and 3, increasing levels of antibodies were detected
from 12 to 26 days after the last plasma exchange (Figs. 2, 3A–B). This rebound effect of antibody production is well known [16]. There
appears to be a correlation between rebound antibodies and antibody
titers [16,25,38,39]. After ABO-mismatched kidney transplantation,
lgM and lgG titers were measured before and after each immunoad-
sorption. If there was a rebound between days — 3 and — 1 pre-
transplantation, or if the titers following the last session exceeded 1/8,
more sessions were required [16].

In the case of patient 4, from the previous patients we had learned the
importance of the rebound antibody production effect and we therefore
monitored the antibody levels both prior to and after transplantation. We
also performed plasma exchange before and after H SCT. This resulted in a
significant decrease in anti-HLA class I antibodies and the patient had a
complete donor engraftment according to chimerism analysis, on day + 23 after HSCT. However, the patient had a slow neutrophil engraftment.
This may have been caused by remaining HLA antibodies, since late engraftment in the CB setting has been found to be correlated with
detection of anti-HLA antibodies before HSCT [40].

The findings in this study and recent previous reports indicate that
antibody-mediated rejection may occur after HSCT. According to
experience from kidney transplantation and from this study, antibodies
that may cause graft failure can be reduced using immune
modulation. The importance of a high cell dose to avoid humoral
rejection after CB transplantation has been shown previously [22]. To
avoid rejection in CB transplantation, it may be beneficial to increase
the cell dose—for example, by giving double CB.

To conclude, if a donor with a negative crossmatch cannot be found
for a patient with antibodies to CD34+/VEGF R2+ or HLA antigens,
immune modulation including plasma exchange and rituximab may
be tried to facilitate engraftment. Further studies are warranted.

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References

[1] Maris M, Storb R. The transplantation of hematopoietic stem cells after non-
myeloablative conditioning: a cellular therapeutic approach to hematologic and


transplantation. Biology of Blood and Marrow Transplantation 2008;14:165−70.

Rejection of parental grafts by resistant F 1 hybrid mice. The Journal of

for a similar or common mechanism for natural killer cell activity and resistance to

allografts by natural killer cells and T cells. Differences in kinetics and target


unrelated, DLA-nonidentical canine marrow grafts is unrestricted by the major


[10] Moeller E. Contact-induced cytotoxicity by lymphoid cells containing foreign

cytotoxicity and antibody-dependent cell-mediated cytotoxicity in patients with
aplastic anemia: distinguishing transfusion-induced sensitization from possible

roles of memory T cells and antidonor immunoglobulin in rejection of allogeneic

et al. Preformed antibody, not primed T cells, is the hybrid and major barrier

immunity is the dominant barrier for allogeneic bone marrow engraftment in

rejections of two consecutive renal allografts and early loss of the third transplant caused by
non-HLA antibodies specific for endothelial cells. Transplant Immunology 1997:5:
321−7.

[16] Tydén G, Kumljen G, Fehrmann I. Successful ABO-incompatible kidney trans-
plantations without spleenectomy using antigen-specific immunoadsorption and


haematopoietic stem cell survival by an internal autocrine loop mechanism.

to the donor stem cell population CD34+/VEGF R2+ are associated with rejection


