Acceptable HLA-mismatching in unrelated donor bone marrow transplantation for patients with acquired severe aplastic anemia

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We retrospectively analyzed the effect of HLA mismatching (HLA-A, -B, -C, -DRB1, -DQB1) with molecular typing on transplantation outcome for 301 patients with acquired severe aplastic anemia (SAA) who received an unrelated BM transplant through the Japan Marrow Donor Program. Additional effect of HLA-DPB1 mismatching was analyzed for 10 of 10 or 9 of 10 HLA allele-matched pairs (n = 169). Of the 301 recipient/donor pairs, 101 (33.6%) were completely matched at 10 of 10 alleles, 69 (23%) were mismatched at 1 allele, and 131 (43.5%) were mismatched at ≥ 2 alleles. Subjects were classified into 5 subgroups: complete match group (group I); single-allele mismatch group (groups II and III); multiple alleles restricted to HLA-C, -DRB1, and -DQB1 mismatch group (group IV); and others (group V). Multivariate analysis indicated that only HLA disparity of group V was a significant risk factor for poor survival and grade II-IV acute GVHD. HLA-DPB1 mismatching was not associated with any clinical outcome. We recommend the use of an HLA 10 of 10 allele-matched unrelated donor. However, if such a donor is not available, any single-allele or multiple-allele (HLA-C, -DRB1, -DQB1) mismatched donor is acceptable as an unrelated donor for patients with severe aplastic anemia. (*Blood.* 2011;118(11):3186-3190)

Introduction

BM transplantation from an unrelated donor (UBMT) is indicated as salvage therapy for patients with severe aplastic anemia (SAA) who fail to respond to immunosuppressive therapy. Early results of UBMT have not been encouraging because of a high incidence of graft failure and GVHD.[1-3] The Center for International Blood and Marrow Transplant Research (CIBMTR) reported the outcome of 232 patients with SAA who received an UBM transplant between 1988 and 1998.[3] The 5-year probabilities of overall survival (OS) were 39% and 36% after matched unrelated and mismatched unrelated donor transplantations, respectively. We previously reported the outcome of 154 patients with SAA who received an UBM transplant between 1993 and 2000 through the Japan Marrow Donor Program (JMDP).[4] The 5-year OS rate was 56% in that study.

In several recent studies, the effect of HLA high-resolution matching on outcome of patients who received an UBM transplant has been elucidated.[5-8] However, results have been derived primarily from an analysis of patients with hematologic malignancies. Major obstacles for UBMT are different between patients with hematologic malignancies and patients with SAA. Relapse is a main cause of death for patients with hematologic malignancies, and GVL effect may result in decrease of relapse rate. In contrast, graft failure is the main problem, and GVHD is the only negative effect for patients with SAA. Therefore, optimal HLA matching may be different between these 2 populations. Algorithms for donor selection derived from an analysis of patients with hemato-

logic malignancies might not be useful for patients with SAA. However, a few studies have focused on the clinical significance of HLA-allele compatibility in patients with SAA.[2,4,9,10]

In a previous study, we analyzed the clinical significance of HLA allele mismatching in 142 patients with SAA, in whom data of high-resolution typing of HLA-A, -B, and -DRB1 were available.[4] Mismatching of HLA-A or -B alleles between donor and recipient was a strong risk factor for acute and chronic GVHD and OS, whereas mismatching of the HLA-DRB1 allele did not have a significant effect on patient outcomes. In the study from the National Marrow Donor Program, mismatching of HLA-DRB1 was the most crucial risk factor for OS.[2] These results indicate that better donor selection through high-resolution typing might result in improved outcome in patients with SAA who receive an UBM transplant. In fact, several recent studies showed a significantly improved outcome in patients with SAA who received and UBM transplant over time.[11,12] In particular, better HLA matching by high-resolution typing has been thought to contribute to these improvements.[4,9-11]

On the contrary, restricting BMT to donor-recipient pairs perfectly matched at high-resolution typing reduces the chance of undergoing UBMT for many patients. Therefore, strategies for selecting a partially HLA allele mismatched donor are required when a full matched donor cannot be identified. Here, we report a detailed analysis of outcome in 301 patients with SAA who were

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- 55 -

typed for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 by a molecular technique and underwent UBMT through the JMDP.

Methods

Patients

From February 1993 to April 2005, 380 consecutive patients with acquired SAA received an UBM transplant through the JMDP. Patients with inherited AA, such as Fanconi anemia, and patients who received a BM transplant > 2 times were excluded. This study includes 301 patients in whom molecular analysis of HLA-A, -B, -C, DRB1, and -DQB1 were performed by DNA-based methods. HLA-DPB1 was analyzed in 299 of these patients. The previous study included 142 patients in whom molecular typing was performed only for HLA-A, -B, and -DRB1.

Characteristics of the 301 patients and donors are shown in Table 1. Briefly, patients (173 males and 128 females) were between birth and 64 years of age (median, 17 years of age). The median disease duration before BMT was 43 months (range, 4-436 months). All patients failed conventional immunosuppressive therapies and were considered candidates for UBMT. All patients or their guardians gave informed consent for transplantation and submission of the data to the JMDP.

Transplantation procedure

Characteristics of the transplantation procedures are also shown in Table 1. Patients underwent transplantations at individual centers following the local protocols for preconditioning regimens and GVHD prophylaxis. The various preconditioning regimens used by individual centers were classified into 5 categories: TBI or LFI + CY + ATG (n = 128), TBI or LFI + CY (n = 103), TBI or LFI + CY + Flu with or without ATG (n = 39), CY + Flu + ATG (n = 8), and others (n = 23). In 130 patients, CsA and MTX were used for prophylaxis against GVHD; 134 patients received FK instead of CsA. The remaining 35 patients received other GVHD prophylaxis. Ex vivo T-cell depletion was not used for any patient. The median number of infused nucleated marrow cells was 3.1×10^8/kg. One-half (n = 150) of the transplantations were performed before 2000, and 151 were done after 2001.

HLA typing and definition of mismatching

HLA matching between patients and donors was based on HLA serotyping according to the standard technique. Partial HLA-A and -B alleles and complete HLA-DRB1 alleles were identified as confirmatory HLA typing during the coordination process, and HLA-A, -B, -C, -DQB1, and -DPB1 alleles were retrospectively reconfirmed or identified after transplantation. Molecular typing of HLA-A, -B, -C, -DQB1, -DRB1, and -DPB1 alleles was performed by the Luminex microbead method (Luminex 100 system) adjusted for the JMDP and in part by the sequencing-based typing method. Mismatching was defined as the presence of donor antigens or alleles not shared by the recipient (rejection vector) or the presence of recipient antigens or alleles not shared by the donor (GVHD vector).

Definition of transplantation-related events

The day of engraftment was defined as the first day of 3 consecutive days on which neutrophil count exceeded 0.5×10^9/L. Patients who did not reach neutrophil counts > 0.5×10^9/L for 3 consecutive days after transplantation were considered to have primary graft failure. Patients with initial engraftment in whom absolute neutrophil counts declined to < 0.5×10^9/L subsequently were considered to have secondary graft failure. Acute GVHD was evaluated according to standard criteria in patients who achieved engraftment, and chronic GVHD was evaluated according to standard criteria in patients who achieved engraftment and survived > 100 days after transplantation.

Data collection and statistical analysis

Transplantation data were collected with the use of standardized forms provided by the JMDP. Patient baseline information and follow-up reports were submitted at 100 days and annually after transplantation. Analysis of patient outcome was performed with the date of last reported follow-up or date of death. Data were analyzed as of July 1, 2007.

Probability of OS and 95% confidence interval (95% CI) were estimated from the time of transplantation according to the Kaplan-Meier method. Cumulative incidence of neutrophil engraftment at day 42 was analyzed in the whole of patients by treating deaths until day 42 as a competing risk. Cumulative incidence of acute GVHD at day 100 was analyzed in patients who sustained engraftment by treating deaths until day 100 as a competing risk. Cumulative incidence of chronic GVHD at day 365 was analyzed in patients who sustained engraftment and survived longer than day 100 by treating deaths until day 365 as a competing risk. In univariate analysis, the log-rank test or Gray test was used to assess the significance of HLA allele mismatching on clinical outcomes. The Mann-Whitney U test was used to compare the median days of neutrophil engraftment. The chi-square test or Mann-Whitney U test was used to compare patient characteristics and transplantation procedures between the patient groups. All P values < .05 were considered statistically significant, whereas P values between .05 and .1 were considered as marginally significant.

Multivariate analyses were performed to assess the effect of HLA allele mismatching on the clinical outcome by Cox proportional hazard model (each mismatched group vs fully matched group; hazard risk = 1.0 as a reference group). Factors other than HLA mismatching included in the models were patient age, patient sex, donor age, donor sex, disease duration before BMT, infused cell dose, matching of ABO blood type, GVHD prophylaxis, and preconditioning regimens.

Results

HLA matching by DNA typing

Of the 301 recipient/donor pairs, 101 pairs (33%) were completely matched at HLA-A, -B, -C, -DRB1, and -DQB1 allele; 69 pairs (23%) were mismatched at 1 HLA allele; 59 pairs (20%) were mismatched at 2 HLA alleles; and 72 pairs (24%) were mismatched at ≥ 3 alleles (Table 2). The number and frequency of 1-allele and 2-allele mismatches in either GVHD or rejection vector or both vectors in each HLA allele were 55 (18.3%) and 7 (2.3%) in HLA-A allele, 32 (10.6%) and 2 (0.7%) in HLA-B allele, 130 (43.2%) and 10 (3.3%) in HLA-C allele, 68 (22.6%) and 5 (1.7%) in HLA-DRB1 allele, 80 (26.6%) and 13 (4.3%) in HLA-DQB1 allele, and 179 (59.5%) and 44 (14.6%) in HLA-DPB1 allele, respectively. Because the frequency of mismatching was too high at the DPB1 allele, analysis of DPB1 mismatching was separated from that of other alleles. In addition, because the number of single-allele mismatched pairs of HLA-A, -B, -C, -DRB1, and -DQB1 were too small for separate analyses, HLA-A and -B were grouped into the mismatch of the HLA-A or HLA-B allele (A/B) and HLA-DRB1 and -DQB1 into the mismatch of the HLA-DRB1 or HLA-DQB1 allele (DRB1/DQB1), respectively.

Survival

Of the 301 patients, 202 are alive at the time of analysis with an observation time from 3 to 128 months (median, 44 months) after transplantation. Five-year OS was 66.3% (95% CI, 60.7%-72.5%) in the whole population (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Subgroup analyses were performed in 8 main subgroups (> 15 recipients) as follows: (1) complete match group (n = 101), (2) single locus (A/B) mismatch group (n = 20), (3) single (C) mismatch group (n = 42), (4) 2 loci (A/B + C) mismatch group (n = 20), (5) 2 loci (DRB1/DQB1) mismatch group (n = 19), (6) 3 loci (A/B + C) mismatch group (n = 15), (7) 3 loci (C + DRB1/DQB1) mismatch group (n = 29), and
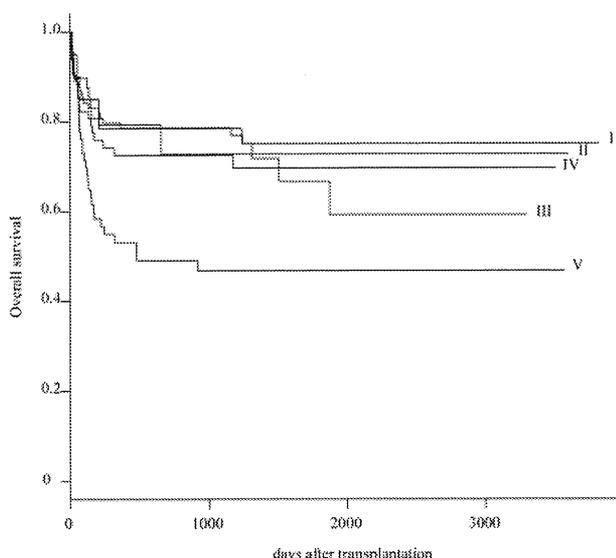
Figure 1. Kaplan-Meier estimates of OS in 5 HLA groups.

(8) 3 loci (A/B + C + DRB1/DQB1) mismatch group (n = 21). OS was significantly worse in the following groups than in the complete match group (75.2%): 2 loci (A/B + C) mismatch group (49.0%; P = .022), ≥ 3 loci (A/B + C) mismatch group (40.0%; P = .002), and A/B + C + DRB1/DQB1 mismatch group (56.1%; P = .031; supplemental Table 1).

On the basis of these primary results, 301 patients were reclassified into 5 subgroups: HLA complete match group (group I; n = 101), single-allele (A/B) mismatch group (group II; n = 20), single-allele (C or DRB1/DQB1) mismatch group (group III; n = 49), multiple-allele (restricted to C or DRB1/DQB1) mismatch group (group IV; n = 68), and others (group V; n = 63). The probability of OS at 5 years was 75.2% (95% CI, 84.8%-66.7%) in group I, 72.7% (95% CI, 96.7%-54.7%) in group II, 66.7% (95% CI, 85.1%-52.3%) in group III, 69.7% (95% CI, 82.6%-58.8%) in group IV, and 46.8% (95% CI, 61.7%-35.5%) in group V, respectively (Table 3; Figure 1). Survival rate was significantly inferior in group V than in group I (P = .003).

To avoid or minimize the effect of other HLA alleles mismatching, the effect of HLA-DPB1 mismatching was evaluated in group I (n = 101) and groups II + III (n = 69), independently. HLA-DPB1 was matched in 51 recipient/donor pairs (30%) and mismatched in 118 pairs (70%). Patient characteristics and transplantation procedures were not different between HLA-DPB1 matched and mismatched groups (supplemental Table 2). The probability of OS at 5 years in group I was equivalent between the HLA-DPB1 matched group (74.4%; 95% CI, 93.2%-59.4%) and the HLA-DPB1 mismatched group (75.7%; 95% CI, 87.2%-65.8%; P = .894; Table 4; Figure 2A). It was also equivalent in groups II + III (71.4%; 95% CI, 93.6%-54.5% in the HLA-DPB1 matched group and in the HLA-DPB1 mismatched group (67.1%; 95% CI, 85.6%-52.5%; P = .826; Table 4; Figure 2B). Multivariate analysis identified significant unfavorable variables as follows: recipient age (0-10 years: relative risk [RR] = 1.0; 11-20 years: RR = 4.092, P = .002; 21-40 years: RR = 3.970, P = .004; > 41 years: RR = 5.241, P = .003), conditioning regimen (Flu + CY + TBI/LFI ± ATG: RR = 1.0; CY + TBI/LFI: RR = 4.074, P = .058; others: RR = 6.895, P = .013), HLA mismatching (group I: RR = 1.0; group V: RR = 1.967, P = .023), donor sex (female: RR = 1.0; male: RR = 1.850, P = .016), and GVHD prophylaxis (FK + MTX: RR = 1.0; other: RR = 1.754, P = .024), blood type

(ABO match or minor mismatch: RR = 1.0; major mismatch or bidirection: RR = 1.948, P = .005), and disease duration (< 7 years: RR = 1.0; > 7 years: RR = 1.540, P = .084; Table 5).

Engraftment

The cumulative incidence of neutrophil engraftment at day 42 was evaluated in 300 patients. It was 90.3% (95% CI, 93.7%-86.9%) in the whole population. Subgroup analyses showed that it was 93.0% (95% CI, 98.2%-87.8%) in group I, 90.0% (95% CI, 100%-74.6%) in group II, 89.8% (95% CI, 98.9%-80.7%) in group III, 92.6% (95% CI, 99.2%-86.0%) in group IV, and 84.1% (95% CI, 93.4%-74.8%) in group V (P = .185; Table 3). The median time to engraftment was 17 days in group I; 18 days in groups II, III, and IV; and 19 days in group V. Engraftment was marginally delayed in group V compared with group I (P = .053). Additional HLA-DPB1 mismatching did not affect the cumulative incidence of engraftment in the 10 of 10 and 9 of 10 matched groups, respectively (Table 4). In multivariate analysis, blood type (ABO match or
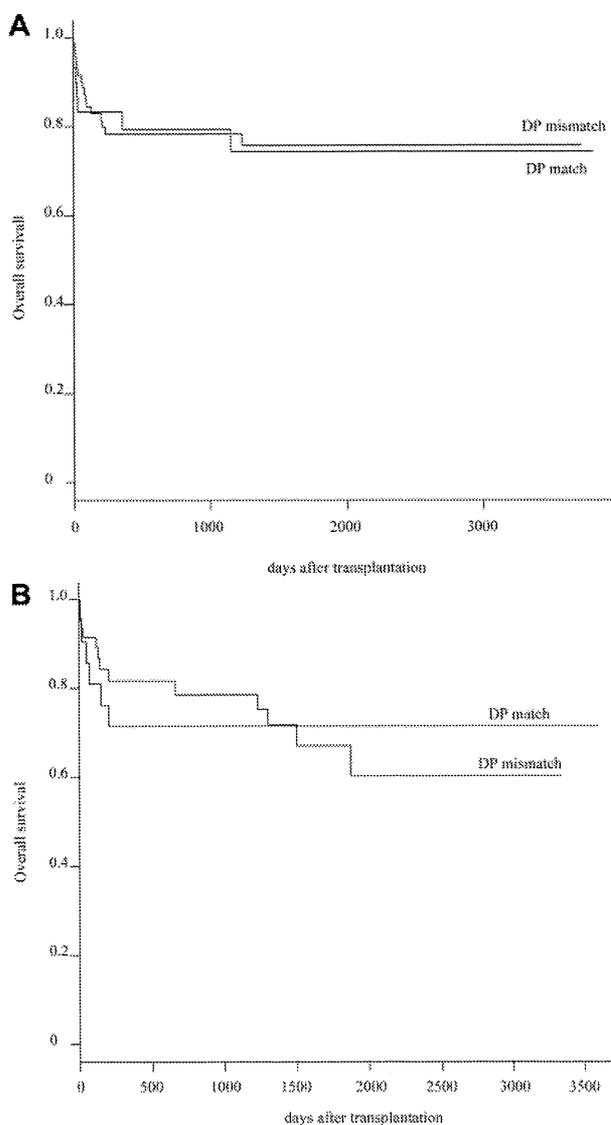

Figure 2. OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group. (A) Difference of OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group in 10 of 10 HLA allele matched pairs. (B) Difference of OS between HLA-DPB1 matched group and HLA-DPB1 mismatched group in 9 of 10 HLA allele matched pairs.
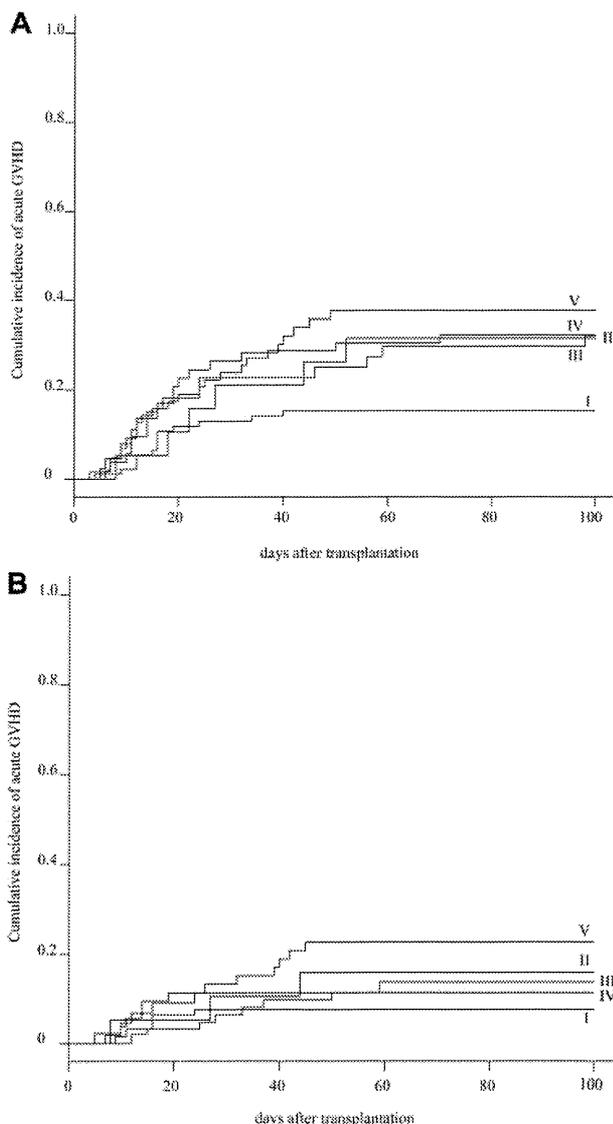
Figure 3. Cumulative incidence of acute GVHD. (A) Cumulative incidence of grade II-IV acute GVHD in 5 HLA groups. (B) Cumulative incidence of grade III-IV acute GVHD in 5 HLA groups.

minor mismatch: RR = 1.0; major mismatch or bidirection pair: RR = 5.102, P = .039) and HLA mismatching (group I: RR = 1.0; group V: RR = 4.906, P = .035) were significant risk factors for engraftment.

Acute GVHD

The cumulative incidence of acute GVHD at day 100 was evaluated in 272 patients. The cumulative incidence of grade II-IV and grade III-IV acute GVHD was 27.2% (95% CI, 32.5%-21.9%) and 12.9% (95% CI, 16.9%-8.9%) in the whole population, respectively (supplemental Figure 2). Subgroup analyses showed that the cumulative incidence of grades II-IV acute GVHD was statistically lower in group I (15.1%; 95% CI, 22.4%-7.8%) than in group V (37.7%; 95% CI, 50.9%-24.5%; P = .037), and marginally lower than in group III (31.8%; 95% CI, 45.8%-17.8%) and group IV (31.7%; 95% CI, 43.3%-20.1%; Table 3; Figure 3A). Whereas the cumulative incidence of grade III-IV acute GVHD was not significantly different among 5 groups: 7.5% (95% CI, 24.6%-0%) in group I, 15.8% (95% CI, 32.7%-0%) in group II, 13.6% (95% CI, 23.9%-3.3%) in group III, 11.1% (95% CI,

18.9%-3.3%) in group IV, and 22.6% (95% CI, 34.0%-11.2%) in group V (P = .139; Table 3; Figure 3B). Additional HLA-DPB1 mismatching evaluated in 155 patients did not affect the cumulative incidence of grade II-IV acute GVHD in the 10 of 10 and 9 of 10 matched groups, respectively (Table 4). Multivariate analysis showed that a significantly higher incidence of grade II-IV acute GVHD was associated with HLA mismatching (group I: RR = 1.0; group III: RR = 3.975, P = .002; group IV: RR = 3.334, P = .004; group V: RR = 3.665, P = .002). Other significant risk factors were the preconditioning regimen (Flu +CY + TBI/LFI ± ATG: RR = 1.0; TBI/LFI + CY: RR = 5.224, P = .003), and donor sex (female: RR = 1.0; male: RR = 1.844, P = .034; supplemental Table 3).

Chronic GVHD

The cumulative incidence of chronic GVHD at day 365 was evaluated in 232 patients. It was 24.5% (95% CI, 30.3%-18.7%) in the whole population. Subgroup analyses showed that it was comparable among the 5 HLA groups: 19.8% (95% CI, 28.8%-10.8%) in group I, 26.3% (95% CI, 49.3%-3.3%) in group II, 28.2% (95% CI, 43.3%-13.1%) in group III, 26.9% (95% CI, 39.2%-14.6%) in group IV, and 27.3% (95% CI, 42.1%-12.5%) in group V (P = .922; Table 3; supplemental Figure 3). HLA-DPB1 mismatching did not affect the cumulative incidence of chronic GVHD (Table 4).

Discussion

The survival rate in UBMT has increased substantially over the past 10 years in patients with SAA.[8-15] A 5-year survival rate of 90% has been reported in a small series of children.[16,17] A recent meta-analysis showed that detailed HLA-matching facilitated by DNA-based typing has contributed to the improved survival rate in patients with SAA who received an UBM transplant.[18] However, many patients with SAA who need hematopoietic stem cell transplantation do not have an HLA-complete matched donor. Our multivariate analysis indicated that among 4 HLA-mismatched groups, only HLA disparity of group V was a statistically significant unfavorable variable. We conclude that any type of HLA single-allele mismatch or multiple-allele mismatch within HLA-C and HLA class II (DRB1 or DQB1) is acceptable as an unrelated donor when an HLA complete match donor is unavailable.

We previously reported that HLA class I allele mismatching (HLA-A or -B) but not class II allele (HLA-DRB1) mismatching was a significant risk factor for survival when 6 alleles were analyzed.[4] HLA-A or -B mismatching pairs in the previous study were separated into 2 groups in the current study in which 10 alleles were analyzed. One group was a true single-allele mismatching pair of HLA-A or -B alleles (group II), and another was a multiple-allele mismatching pair of HLA-A or -B plus HLA-C and/or class II HLA alleles (group V). Because HLA-C and -DQB1 alleles were not typed, this type of multiple-allele mismatching might be mistaken as a single-allele mismatching pair, which was the reason for the inferior outcome of HLA-class I mismatching pairs in our previous study.

As the same in our previous study, mismatching of HLA-DRB1 did not provide a significant impact on clinical outcome. An HLA-DRB1 mismatching pair was also classified into a true single-allele mismatching of HLA-DRB1 (group III) and HLA-DRB1 plus HLA-C and/or HLA-DQB1 mismatching pairs (group IV). Interestingly, multiple mismatching of group IV was not

associated with increased mortality, which may explain why mismatching of HLA-DRB1 did not have a deleterious effect in the previous study.

The effect of HLA-DPB1 mismatching was also evaluated in HLA complete matched pairs (n = 101) and single-allele mismatched pairs (n = 69). The importance of DPB1 matching in the UBMT setting has been mainly discussed in patients with hematologic malignancies. Although results were controversial in early reports, recent studies support a significant effect of DPB1 mismatching on the incidence of acute GVHD, disease relapse, and OS.[19-22] In a large dataset of the International Histocompatibility Working Group, there was a statistically significant higher risk of both grade II-IV and grade III-IV acute GVHD.[19] The increased risk of acute GVHD was accompanied by a statistically significant decrease in disease relapse, probably because of the GVL effect, which offset the deleterious effect of acute GVHD. Survival rate was significantly better in DPB1-matched transplantations in patients with standard-risk leukemia but not in advanced leukemia. Conversely, in the HLA-mismatched group, there was a significant survival advantage in DPB1 mismatched pairs.

We expected that DPB1 matching might be beneficial for patients with AA who do not need the GVL effect. However, clinical outcomes, including incidence of acute GVHD, were not affected by DPB1 mismatching. HLA-DPB1 typing may not be essential to the donor selection algorithm for patients with SAA.

Indeed, HLA-DPB1 mismatching was observed in 74% of recipient/donor pairs, and it may be practically difficult to find HLA 12 of 12 matched donors.

In conclusion, this retrospective study confirms the importance of HLA matching between recipients and donors to improve the outcome of UBMT for patients with SAA patients. However, this study showed that only 33% of patients received transplants from an HLA 10 of 10 matched donor. The availability of unrelated hematopoietic stem cell transplants can be increased through the judicious selection of donors with HLA mismatches that do not substantially lower survival.

Authorship

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Original Articles

Relapse of aplastic anemia in children after immunosuppressive therapy: a report from the Japan Childhood Aplastic Anemia Study Group

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ABSTRACT

Background

Although the therapeutic outcome of acquired aplastic anemia has improved markedly with the introduction of immunosuppressive therapy using antithymocyte globulin and cyclosporine, a significant proportion of patients subsequently relapse and require second-line therapy. However, detailed analyses of relapses in aplastic anemia children are limited.

Design and Methods

We previously conducted two prospective multicenter trials of immunosuppressive therapy for children with aplastic anemia: AA-92 and AA-97, which began in 1992 and 1997, respectively. In this study, we assessed the relapse rate, risk factors for relapse, and the response to second-line treatment in children with aplastic anemia treated with antithymocyte globulin and cyclosporine.

Results

From 1992 to 2007, we treated 441 children with aplastic anemia with standard immunosuppressive therapy. Among the 264 patients who responded to immunosuppressive therapy, 42 (15.9%) relapsed. The cumulative incidence of relapse was 11.9% at 10 years. Multivariate analysis revealed that relapse risk was significantly associated with an immunosuppressive therapy regimen using danazol (relative risk, 3.15; $P=0.001$) and non-severe aplastic anemia (relative risk, 2.51; $P=0.02$). Seventeen relapsed patients received additional immunosuppressive therapy with antithymocyte globulin and cyclosporine. Eight patients responded within 6 months. Seven of nine non-responders to second immunosuppressive therapy received hematopoietic stem cell transplantation and five are alive. Eleven patients underwent hematopoietic stem cell transplantation directly and seven are alive.

Conclusions

In the present study, the cumulative incidence of relapse at 10 years was relatively low compared to that in other studies mainly involving adult patients. A multicenter prospective study is warranted to establish optimal therapy for children with aplastic anemia.

Key words: children, aplastic anemia, relapse, risk factors, immunosuppressive therapy.

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Introduction

Aplastic anemia (AA) is thought to be an immune-mediated bone marrow disease, characterized by bone marrow aplasia and peripheral blood pancytopenia. Currently, two effective treatments are available for this disorder: allogeneic bone marrow transplantation and immunosuppressive therapy. Bone marrow transplantation from a human leukocyte antigen (HLA)-matched sibling donor can cure the majority of transplanted patients with severe AA.[1] The outcome after bone marrow transplantation has been markedly better in children than in adults, with less frequent and severe graft-versus-host disease and better overall survival.[2,3] However, most children with severe AA have no matched sibling donor and rely on immunosuppressive therapy as first-line treatment.

The combination of antithymocyte globulin and cyclosporine is now considered the standard immunosuppressive regimen for children with severe AA who lack a matched sibling donor.[4] Recent large trials of combined immunosuppressive therapy for severe AA in children demonstrated that the response rate is greater than 60% and the 3- to 5-year survival rate is approximately 90%.[5-7] However, relapse and clonal evolution with transformation to myelodysplasia or acute myeloid leukemia remain significant problems after immunosuppressive therapy, and long-term, event-free survival is less impressive than after bone marrow transplantation.[4,8] We previously reported the results of a multicenter trial of immunosuppressive therapy for children with AA (AA-92 study).[5] In the AA-92 study, the response rate at 6 months was 71%, with the probability of survival at 4 years being greater than 90%. However, a significant proportion of patients subsequently relapsed and required second-line therapy. To select the optimal therapy for such patients, a detailed analysis concerning relapse after response to immunosuppressive therapy is very important; however, analyses of relapse of AA in children after the standard combined immunosuppressive regimen are very limited.[9-11] Although the European Group for Blood and Marrow Transplantation (EMBT) reported an analysis of relapse of AA after immunosuppressive therapy in a large number of patients, the study populations were primarily adults treated in the 1970s and 1980s with antithymocyte globulin monotherapy.[9] A report from the Italian Association of Pediatric Hematology and Oncology focused mainly on the response to cyclosporine and dependence after immunosuppressive therapy.[10] A single-center retrospective analysis from the National Institutes of Health showed excellent long-term survival with a 33% cumulative incidence of relapse at 10 years in children with severe AA who responded to the standard immunosuppressive therapy; however, a detailed analysis of relapse that included risk factors was not provided.[11]

We previously conducted two prospective multicenter studies: the AA-92 and AA-97, which began in November 1992 and October 1997, respectively.[5,12] From 1992 to 2007, 473 children with AA were treated with immunosuppressive therapy in these studies, and 441 of the children were treated with antithymocyte globulin plus cyclosporine. In the present study, we assessed the relapse rate, risk factors for relapse, response to second-line treatment, and prognosis after relapse in AA children treated with an antithymocyte globulin/ cyclosporine-based regimen.

Design and Methods

Patients

Two consecutive prospective studies were designed by the Japan Childhood Aplastic Anemia Study Group and involved 79 hospitals in Japan. The eligibility criteria have been described previously.[5] The severity of disease was determined according to currently used criteria.[13,14] Disease was considered severe if at least two of the following were present: (i) neutrophil count less than 0.5×10^9/L; (ii) platelet count less than 20×10^9/L; and (iii) reticulocyte count less than 20×10^9/L with a hypocellular bone marrow. AA was considered very severe if the above criteria for severe disease were fulfilled and the neutrophil count was less than 20×10^9/L. Non-severe disease was defined by at least two of the following: (i) neutrophil count less than 1.0×10^9/L, (ii) platelet count less than 50×10^9/L; and (iii) reticulocyte count less than 60×10^9/L with a hypocellular bone marrow. Allogeneic bone marrow transplantation was recommended for those patients with severe or very severe disease who had a matched sibling donor. This study was approved by the Ethic Committee of Hyogo Children Hospital.

Treatment

The details of the immunosuppressive therapy administered were described in previous reports.[5,12] Immunosuppressive therapy consisted of horse antithymocyte globulin (Lymphoglobulin; Genzyme Corp., Cambridge, MA, USA) (15 mg/kg per day, days 1 to 5), cyclosporine (6 mg/kg per day, days 1 to 180, with subsequent adjustments to maintain the whole blood cyclosporine concentration between 100 to 200 ng/mL), and methylprednisolone for prophylaxis against allergic reactions (2 mg/kg per day for 5 days, with subsequent halving of the dose every week until discontinuation on day 28). Patients with very severe AA were treated with immunosuppressive therapy plus granulocyte-colony stimulating factor (G-CSF) (Filgrastim; Kirin, Tokyo, Japan) [400 μg/m^2 on day 1, with responding patients (neutrophil count > 1.0×10^9/mL) receiving the same dose three times a week for 3 months in the AA-92 study and for 60 days in the AA-97 study]. In the AA-92 study, the addition of G-CSF to immunosuppressive therapy for patients with severe AA and non-severe AA was randomized, while in the AA-97 study, G-CSF was not given to patients with severe AA or non-severe AA except to those with documented severe infection. All patients in the AA-92 study received danazol at a dose of 5 mg/kg/day for 6 months, and danazol was discontinued without tapering.

Assessments

A complete response was defined for all patients as a neutrophil count greater than 1.5×10^9/L, a platelet count greater than 100×10^9/L, and a hemoglobin level greater than 11.0 g/dL. For patients with severe AA and very severe AA, a partial response was defined as a neutrophil count greater than 0.5×10^9/L, a platelet count greater than 20×10^9/L, a hemoglobin level greater than 8.0 g/dL, and no requirement for blood transfusions. For patients with non-severe AA, a partial response was defined as a neutrophil count greater than 1.0×10^9/L, a platelet count greater than 30×10^9/L, a hemoglobin level greater than 8.0 g/dL, and no requirement for blood transfusions.[5] In patients with a complete response on day 180, the cyclosporine dose was tapered down slowly (10% of adjusted dose per month). In those with a partial response, cyclosporine was continued for another 6 months to allow further improvement of blood counts. Tapering of cyclosporine was started on day 360 (10% every 2 weeks) regardless of response.

The hematologic response was evaluated 6 months after the

initiation of therapy. Relapse was defined by conversion to no response from a partial or complete response and/or the requirement for blood transfusions.[5]

Statistical analysis

Failure-free survival curves were calculated by the Kaplan-Meier method, and evaluated by the log-rank test. The Cox proportional hazards model was used to assess the risk factors for relapse after immunosuppressive therapy using both univariate and multivariate analyses. The estimated magnitude of the relative risk (RR) is shown along with the 97.5% confidence interval (CI). Cumulative incidence using the competing risk method, as described by Fine and Gray,[15] was used for the assessment of factors predicting relapse. The competing events of relapse were death and transplantation.

Results

Patients' characteristics

In the AA-92 and AA-97 studies, 441 AA children were treated with antithymocyte globulin plus cyclosporine between 1992 and 2007. The characteristics of all the patients studied are summarized in Table 1. There were 112 and 329 patients in the AA-92 and AA-97 studies, respectively. The median age of all these patients was 8.3 years (range, 0 to 17 years). Patients with very severe (n=210), severe (n=149) and non-severe disease (n=82) received initial immunosuppressive therapy consisting of antithymocyte globulin and cyclosporine. Six months after the initial immunosuppressive therapy, 264 patients (59.9%) had achieved a complete response (n = 91) or partial response (n=173). Among the 264 patients who responded to immunosuppressive therapy, 42 (15.9%) subsequently relapsed. The cumulative incidence of relapse was 11.9% at 10 years and the median time from diagnosis to relapse was 21 months (range, 6 to 138 months). The median time from response to antithymocyte globulin therapy to relapse was 22 months (range, 2 to 135 months).

Risk factors for relapse

Two hundred and sixty-four patients with a total of 42 events were eligible for analyses of risk factors for relapse. In univariate analysis, two parameters, non-severe disease (RR=2.98, 97.5% CI 1.40 - 6.34, P=0.0047) and use of danazol (RR=3.44, 97.5% CI 1.78 - 6.65, P=0.00023), were statistically significant risk factors (Table 2). In contrast, the relative risk of relapse for patients with post-hepatitis AA was significantly lower than the relative risk for patients with idiopathic AA (RR=0.234, P=0.043). Gender, age, duration of AA prior to initial treatment, early response (within 90 days after immunosuppressive therapy), use of G-CSF, and HLA-DR2 could not be identified as risk factors. In multivariate analysis, two factors, non-severe AA (RR=2.51, 97.5% CI 1.15 - 5.46, P=0.02) and use of danazol (RR=3.15, 97.5% CI 1.62 - 6.12, P=0.001) remained statistically significant. Figure 1A shows the cumulative incidence of relapse of patients with non-severe AA (35.3%), severe AA (12.9%), and very severe AA (12.0%) 10 years after the first immunosuppressive therapy. The cumulative relapse rate of patients with non-severe AA was significantly higher than that of patients with severe AA (P=0.025) or very severe AA (P=0.005). Figure 1B shows the actuarial risk of relapse at 10 years

among patients treated with danazol (29.0%) and in the group not treated with danazol (9.8%) (P<0.001).

Repeated immunosuppressive therapy versus hematopoietic stem cell transplantation as second-line therapy

Among 42 relapsed patients, 17 received a second course of immunosuppressive therapy with antithymocyte globulin and cyclosporine. Eight of these 17 patients responded within 6 months and are alive. Seven of nine non-responders to second immunosuppressive therapy received hematopoietic stem cell transplantation (HSCT) as salvage therapy. The hematopoietic stem cell donors were HLA-matched unrelated bone marrow donors (n=4), unrelated cord blood donors (n=2) and one matched sibling donor. Five of seven patients are alive following HSCT. Eleven patients underwent HSCT directly from an alternative donor (unrelated bone marrow donor, n=7; unrelated cord blood donor, n=1, HLA-mismatched family donor, n=3) and seven are alive. The estimated failure-free survival from the beginning of second-line therapy was 63.6% in the HSCT group compared with 47.1% in the groups treatment with repeated immunosuppressive therapy (P=0.96).

Table 1. Patients' pretreatment characteristics.

	Very severe AA	Severe AA	Non-severe AA
Registered	210	149	82
Sex (male/female)	115/95	83/66	47/35
Median age, years (range)	8.1 (0-17)	8.3 (1-17)	8.5 (2-16)
Etiology of AA			
Ideopathic	168	125	74
Hepatitis	37	21	7
Viral infection	2	1	0
Drug	3	2	1
Median days from diagnosis to treatment (range)	20.4 (1-146)	30.6 (1-180)	44.8 (3-180)
Study (AA-92/AA-97)	46/164	38/111	28/54
Response (complete/ partial) (%)	128 (40/88) (61.0%)	91 (38/53) (61.1%)	45 (13/32) (54.9%)
Relapse (AA-92/AA-97)	6/8	9/5	11/3

Table 2. Risk factors for relapse in patients with aplastic anemia by univariate analysis.

| Variable	Relative risk (97.5% CI)	P
Sex, male	0.977 (0.514-1.86)	0.94
Age	1.01 (0.947-1.08)	0.78
Etiology of AA		
Ideopathic	4.97 (1.22-20.2)	0.025
Hepatitis	0.234 (0.0577-0.952)	0.043
Duration of AA prior to initial treatment	1.01 (0.998-1.02)	0.11
Response at 90 days	1.07 (0.517-2.21)	0.86
Severity of disease		
Non-severe	2.98 (1.40-6.34)	0.0047
Severe	1.21 (0.561-2.63)	0.62
Very severe	1	
Study, AA-92 (Danazol+)	3.44 (1.78-6.65)	0.00023
G-CSF (+)	0.915 (0.363-2.31)	0.85
HLA-DR2	0.905 (0.307-2.67)	0.86
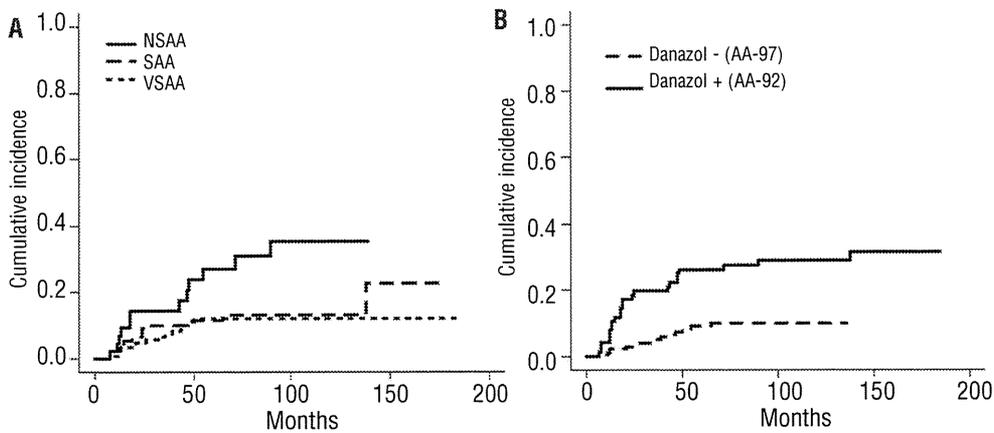
Figure 1. Cumulative incidence of relapse after immunosuppressive therapy in children with aplastic anemia. (A) The cumulative relapse rate of patients with non-severe aplastic anemia (NSAA) was significantly higher than that of patients with severe aplastic anemia (SAA) (*P*=0.025) and very severe aplastic anemia (VSAA) (*P*=0.005) 10 years after the first immunosuppressive therapy. (B) The actuarial risk of relapse at 10 years was significantly higher in the group treated with danazol (29.0%) than in the group not treated with danazol (9.8%) (*P*<0.001).

The overall survival rate did not differ between the immunosuppressive therapy group (84.7%) and the HSCT group (63.6%) after second-line treatment (*P*=0.07). Other patients were treated with cyclosporine alone (n=6) or bone marrow transplantation from a matched sibling donor (n=6). Two patients did not receive second-line treatments. One patient developed clonal evolution to myelodysplastic syndrome after 65 months, and the second developed acute myeloid leukemia after 37 months. Two patients showed clonal evolution to paroxysmal nocturnal hemoglobinuria after 138 months and 55 months. There were seven deaths among the 42 patients who initially relapsed. The causes of death were HSCT-related complications (n=5), acute myeloid leukemia (n=1) and bacteremia (n=1). The overall 10-year survival rates for patients with very severe AA, severe AA, and non-severe AA were 82.2±3.3%, 82.1±4.7% and 98.2±1.8%, respectively.

Discussion

Analysis of relapse in children with AA responding to immunosuppressive therapy will provide valuable information for the management of childhood AA. Here, we present the results of a comprehensive analysis of the largest consecutive series of AA children treated with standard immunosuppressive therapy. Relapse of AA after immunosuppressive therapy is relatively common, with actuarial risks of 30 - 40% having been reported.[16-18] In the present study, the cumulative incidence of relapse at 10 years was 11.9%, which is relatively low compared with that found in other studies that primarily involved adult patients.[16-18] Differences in the study populations may explain the discrepancy between the results of our current study and those of the other studies. A recent Italian study of childhood AA showed a 16% cumulative incidence of relapse, which is comparable with that found in our study.[10]

Multivariate analysis of the data from this retrospective multicenter study shows that the use of danazol was the most statistically significant risk factor for relapse. From 1992 to 2007, 441 children with newly diagnosed AA were treated with immunosuppressive therapy consisting of antithymocyte globulin and cyclosporine with (the AA-92 study) or without danazol (the AA-97 study). There are several reports of the efficacy of anabolic steroids in the treatment of AA. A randomized trial from the EBMT SAA working party demonstrated that the addition of an ana-

bolic steroid (oxymetholone) to antithymocyte globulin treatment improved the response rate of patients with treated AA.[14] In our study, consistent with that report, the response rate at 6 months was higher in the patients who received immunosuppressive therapy with danazol (67.9%) than in the group of patients who received immunosuppressive therapy without danazol (57.1%). Furthermore, our results also showed that the cumulative relapse rate was significantly higher in the patients treated with immunosuppressive therapy plus danazol (Figure 1B). The reason danazol has an impact on relapse is unknown. However, it is possible that a number of cases with an androgen-responsive congenital bone marrow failure syndrome such as dyskeratosis congenita were hidden in our series of AA patients, and discontinuation of danazol was responsible for relapse. Recent reports have shown that a bone marrow failure syndrome of variable severity due to dyskeratosis congenita may be present in otherwise phenotypically normal individuals, and can masquerade as acquired AA.[19-22] We found mutations in the telomerase reverse transcriptase (*TERT*) gene, which is one of the genes causing dyskeratosis congenita, in two of 96 Japanese children with acquired AA.[23] Recently, more dyskeratosis congenita genes have been discovered. It is possible that more cases with an androgen-responsive dyskeratosis congenita were hidden in our series of AA patients. Alternatively, danazol may inhibit complete eradication of pathological T-cell clones by antithymocyte globulin through an unknown mechanism. Understanding the effects of androgens and developing androgen-mimetic drugs could be of significant benefit.

In our cohort of patients with non-severe AA, most patients were transfusion-dependent. In the AA-92 and AA-97 studies, 82 patients with non-severe AA were treated with the standard immunosuppressive regimen consisting of antithymocyte globulin and cyclosporine. Six months after the initial immunosuppressive therapy, 13 patients had achieved a complete response and 32 patients achieved a partial response. Among the 32 patients who achieved a partial response, 14 patients later relapsed. However, 18 patients with non-severe AA patients who achieved a partial response maintained their hematologic response, and 12 of them subsequently achieved a complete response. When childhood non-severe AA is treated with supportive care, 67% of patients progress to develop severe AA, suggesting that it is important to consider early immunosuppressive therapy.[24] Our data indicate that

immunosuppressive therapy is beneficial for some patients with non-severe AA.

A previous Japanese study showed that the addition of G-CSF to immunosuppressive therapy increased the hematologic response rate after 6 months and reduced the relapse rate in adult patients with severe AA.[25] Recently, Gurion et al. conducted a systematic review and meta-analysis of randomized controlled trials comparing treatments with immunosuppressive therapy with or without hematopoietic growth factors in patients with AA. The addition of hematopoietic growth factors did not affect mortality, response rate, or occurrence of infections, but did significantly decrease the risk of relapse.[26] The data from our AA-92 trial were included in this meta-analysis. In contrast to the other five studies in the meta-analysis, only our study included patients with non-severe AA, who had a significantly higher relapse rate than that of patients with either severe AA or very severe AA. Differences in the study populations may explain the discrepancy between the results of our current study and those of the other studies in the meta-analysis. To compare our results with the other studies, we excluded patients with non-severe AA from the statistical analysis, and compared the risk of relapse between patients who did or did not receive G-CSF. The results again showed no significant differences in the relative risk between them (RR=2.71, 97.5% CI 0.614 - 12.0, P=0.19).

The majority of patients who experienced relapse responded to reintroduction of immunosuppressive agents.[27] Our present study also demonstrates that a second course of immunosuppressive therapy was a safe and effective treatment for the patients who relapsed after the first immunosuppressive therapy. However, an optimal second immunosuppressive therapy regimen has not yet been established. Furthermore, about half of the relapsing patients eventually received HSCT in our study. The treatment choice was based on center-related preferences or on anecdotal evidence. A multicenter prospective study is warranted to establish optimal therapy for these patients.

Appendix

The following centers and persons participated in the Japan Childhood Aplastic Anemia Study Group: Japanese Red Cross Nagoya First Hospital (K. Kato); Kyoto Prefectural University of Medicine (S. Morimoto); Kobe University School of Medicine (Y. Takeshima); Hyogo College of Medicine (Y. Ohtsuka); Tokai University (H. Yabe); Shizuoka Children's Hospital (J. Mimaya); Fukushima Medical University (A. Kikuta); Tokyo Metropolitan Children's Medical Center, Tokyo (T. Kaneko); Osaka City General Hospital (J. Hara); Nagoya University (S. Kojima) ; Jichi Medical School (T. Yamauchi); Kagoshima University (Y. Kawano); Okayama University (M. Oda); Hokkaido University (R. Kobayashi); Hiroshima University (S. Nishimura); Kanazawa University (S. Koizumi); Keio University (T. Mori); Hiroshima Red Cross Atomic Bomb Hospital (K. Hamamoto); Chiba University (T. Sato); Hirosaki University (E. Ito); Teikyo University School of Medicine (F. Ohta); Tottori University (T. Kawakami); Dokkyo University School of Medicine (K. Sugita); Kumamoto National Hospital (K. Takagi); Seirei Hamamatsu Hospital (T. Matsubayashi); Hyogo Children's Hospital (Y. Kosaka); Yokohama City University (K. Ikuta); Yamaguchi University (H. Ayukawa); Kanagawa Children's Medical Center (T. Kigasawa); Hirakata City Hospital (C. Kawakami); Nakadohri General Hospital (A. Watanabe); Gumma Children's Hospital (T. Shitara); National Defence Medical College (I. Sekine); Gifu University School of Medicine (K. Isogai); Kumamoto University School of Medicine (S. Morinaga); University of Ryukyu (N. Hyakuna); Narita Red Cross Hospital (K. Sunami); Asahikawa Medical College (M. Yoshida); Nagoya City University (Y. Ito).

Authorship and Disclosures

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- 65 -

Predicting response to immunosuppressive therapy in childhood aplastic anemia

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ABSTRACT

In aplastic anemia, predictive markers of response to immunosuppressive therapy have not been well defined. We retrospectively evaluated whether clinical and laboratory findings before treatment could predict response in a pediatric cohort from the multicenter AA-97 study in Japan. Between 1997 and 2006, 312 newly diagnosed children were enrolled and treated with a combination of antithymocyte globulin and cyclosporine. In multivariate analyses, lower white blood cell count was the most significant predictive marker of better response; patients with white blood cell count less than 2.0×10^9/L showed a higher response rate than those with white blood cell count of 2.0×10^9/L or more ($P=0.0003$), followed by shorter interval between diagnosis and therapy ($P=0.01$), and male sex ($P=0.03$). In conclusion, pre-treatment clinical and laboratory findings influence response to therapy. The finding that response rate worsens with increasing interval between diagnosis and treatment highlights the importance of prompt immunosuppressive therapy for patients with aplastic anemia.

Key words: aplastic anemia, children, immunosuppressive therapy, predictive marker.

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Introduction

Aplastic anemia (AA) is defined as peripheral blood pancytopenia caused by bone marrow failure, and the pathogenesis is thought to involve autoimmune processes.[1-3] Several studies have confirmed immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and cyclosporine (CyA) as a promising therapeutic option for patients lacking HLA-identical related donors.[4-8] Although several potential markers of IST response that appear to reflect the immune pathophysiology of aplastic anemia have been suggested, mainly from adult studies,[9-11] none have been widely accepted. We have already investigated the clinical relevance of HLA, a minor population of paroxysmal nocturnal hemoglobinuria-type cells, and a specific autoantibody associated with aplastic anemia in pediatric patients, finding no correlation between these markers and response to therapy.[12]

Some groups have recently shown that pre-treatment laboratory variables are associated with good response to immunosuppressive therapy, but those results remain controversial, as the numbers of children included in the study was relatively small and the drugs used for immunosuppressive therapy have not been consistent.[13-15] The present study, therefore, evaluated whether clinical and laboratory findings before treatment could predict immunosuppressive therapy response in a large population of children with aplastic anemia enrolled in a multi-center study.

Design and Methods

Patients

Between October 1997 and September 2006, a total of 312 Japanese children with aplastic anemia (AA) from 118 hospitals were enrolled in the AA-97 multicenter study conducted by the Japan Childhood Aplastic Anemia Study Group. Patients with acquired AA were eligible if the following criteria were met: age under 18 years; newly diagnosed disease (≤180 days) without specific prior treatment; and moderate to very severe AA. The disease was considered severe if at least 2 of the following were noted: neutrophil count less than 0.5×10^9/L; platelet count less than 20×10^9/L; or reticulocyte count less than 20×10^9/L with hypocellular bone marrow.[16] AA was considered very severe if the criteria for severe disease were fulfilled and neutrophil count was less than 0.2×10^9/L. Moderate disease was defined by at least 2 of the following: neutrophil count less than 1.0×10^9/L; platelet count less than 50×10^9/L; or reticulocyte count less than 60×10^9/L.[6] Patients with congenital AA or paroxysmal nocturnal hemoglobinuria were excluded. Allogeneic stem cell transplantation was recommended for patients with severe or very severe disease who had an HLA-matched sibling, so these patients were not included in the AA-97 study. Written informed consent was obtained from all parents and all patients over the age of ten years. All study protocols were approved by the ethics committee of each participating hospital. The study also conforms to the recently revised Declaration of Helsinki.

- 66 -

IST

All patients were treated with a combination of intravenous ATG (Lymphoglobulin; Genzyme, Cambridge, USA) at 15 mg/kg/day for five days and oral CyA at 6 mg/kg/day. The dose of CyA was adjusted to maintain trough levels between 100 and 200 ng/mL, and the appropriate dose was administered for at least six months. Granulocyte colony-stimulating factor (Filgrastim; Kirin, Tokyo, Japan) was administered intravenously or subcutaneously at 400 μg/m² for three months only to patients with very severe disease.[17] Response to IST was evaluated at six months after initiation of therapy. Complete response (CR) was defined as a neutrophil count more than 1.5×10⁹/L, a platelet count more than 100×10⁹/L, and a hemoglobin level more than 11.0 g/dL.[17] Partial response (PR) was defined as a neutrophil count more than 0.5×10⁹/L, a platelet count more than 20×10⁹/L, and a hemoglobin level more than 8.0 g/dL in patients with severe or very severe AA, and as a neutrophil count more than 1.0×10⁹/L, a platelet count more than 30 ×10⁹/L, and a hemoglobin level more than 8.0 g/dL in patients with moderate AA.[17] Overall response was defined as CR or PR at six months after IST.

Statistical analyses

Parameters for univariate analyses to determine predictors of response to IST included age at diagnosis, sex, interval between diagnosis and treatment, etiology, severity of disease, white blood cell (WBC) count, neutrophil count, lymphocyte count, hemoglobin level, reticulocyte count, and platelet count. Pre-treatment laboratory values were defined as the lowest value without transfusions during the four weeks preceding IST. Continuous variables were divided into quartile categories, and these cut offs were used for categorical analysis. To evaluate correlations between these parameters and response, differences in continuous variables were analyzed using the Mann-Whitney U-test and differences in frequencies were tested using the χ^2 or Fisher's exact test. For multivariate analyses, logistic regression modeling was performed. Important covariates in the multivariate models were chosen using stepwise variable selection procedures. Values of $P<0.05$ were considered statistically significant.

Results and Discussion

Patients' characteristics are shown in Table 1. A total of 312 patients fulfilled the eligibility criteria. Median age at diagnosis was eight years. Severity of AA was considered very severe in 156 patients, severe in 107 patients, and moderate in 49 patients. The median interval between diagnosis and treatment was 15 days. A total of 176 of the 312 (56.4%) patients improved with IST and achieved PR (n=131) or CR (n=45) at six months. All of them achieved transfusion independence.

To determine predictors of IST response, we compared differences in potential pre-treatment variables between IST responders and non-responders. The following were analyzed both for prevalence in categorical variables and differences in continuous variables: age at diagnosis, interval between diagnosis and treatment, WBC count, neutrophil count, lymphocyte count, hemoglobin level, reticulocyte count, and platelet count. In univariate analyses, WBC count, lymphocyte count, interval between diagnosis and therapy, and gender showed associations with IST response (Table 2). We also performed multivariate logistic regression analysis to assess the simultaneous contributions of each of the variables in predicting response. In these analyses, lower WBC count ($P=0.0003$), shorter interval

between diagnosis and therapy ($P=0.012$), and male sex ($P=0.036$) represented significant predictors of better response (Table 2).

Boys displayed better response than girls (Figure 1A). This relationship was also observed in a retrospective European study in which a young female cohort experienced delayed recovery of bone marrow function following IST.[18] Median WBC count before treatment was significantly lower in patients who achieved response (1.9×10⁹/L) than in those who did not (2.3×10⁹/L; $P= 0.007$). In addition to the analysis with continuous variable, lower WBC count according to categorical analysis also associated with favorable response, with 93 of 144 patients (65%) with WBC less than 2.0×10⁹/L and 83 of 168 patients (49%) with WBC of 2.0×10⁹/L or more showing improvement with IST ($P=0.009$; Figure 1B). When lymphocyte count was applied to the analysis instead of WBC count, a correlation between lower lymphocyte count and response to IST was also observed (Table 2); 82 of 123 patients (67%) with lymphocyte count less than 1.5×10⁹/L improved with IST, a significantly higher frequency than the 94 of 189 patients (50%) with lymphocyte count of 1.5×10⁹/L or more who improved with IST ($P=0.004$). Neither neutrophil count nor severity of disease was predictive of response.

Regarding the association between pre-treatment neutrophil count and response, conflicting results have been reported. A European study reported superior response rates in children with very severe AA compared to severe AA[5] but, in contrast, some studies including a recent report of a Korean cohort of adult patients have produced the opposite results.[13,19] The present findings differ from those published studies, with favorable responses correlating well with lower WBC count rather than neutrophil count or disease severity. Indeed, WBC count was the strongest predictor of response to IST in multivariate analysis. In patients with AA, pre-treatment WBC count may mainly reflect the size of lymphocyte populations, due to the severe neutropenia in this condition. These results suggest that poor response to IST might possibly be ascribed to higher WBC

Table 1. Patients' characteristics.

N. of patients		312
Age at diagnosis, years, median (range)		8 (1-17)
Gender	male / female	186 /126
Etiology	n. of patients (%)	
Idiopathic		261 (83.7)
Hepatitis		44 (14.1)
Others		7 (2.2)
Severity of AA	n. of patients (%)	
VSAA		156 (50.0)
SAA		107 (34.3)
MAA		49 (15.7)
Peripheral blood data at diagnosis		
Median WBC count, ×10⁹/L (range)		2.02 (0.20-8.70)
Median neutrophil count, ×10⁹/L (range)		0.22 (0.00-3.13)
Median lymphocyte count, ×10⁹/L (range)		1.82 (0.10-8.50)
Median Hb level, g/dl (range)		6.9 (2.1-13.2)
Median reticulocyte count, ×10⁹/L (range)		16.0 (0.0-98.0)
Median platelet count, ×10⁹/L (range)		11.0 (1.0-109.0)
Interval from diagnosis to treatment, days, median (range)		15 (1-180)

VSAA: very severe aplastic anemia; SAA: severe aplastic anemia; MAA: moderate aplastic anemia; WBC: white blood cell; Hb: hemoglobin.

- 67 -

Table 2. Univariate and multivariate analysis for IST response in 312 patients with AA.

Univariate variables	Responder	Non-responder	P
N. of patients (%)	176 (56.4)	136 (43.6)	
Median age at diagnosis, years	8	8	NS
Gender, male / female	115/61	71/65	0.025
Etiology, n. of patients (%)			
Idiopathic	141(80)	120(88)	NS
Hepatitis	29(17)	15(11)	
Others	6(3)	1(1)	
Severity of AA, n. of patients (%)			
VSAA	90(51)	66(49)	NS
SAA	62(35)	45(33)	
MAA	24(14)	25(18)	
Median WBC count, ×10⁹/L	1.900	2.255	0.007
≥ 2.0×10⁹/L, n. of patients (%)	87(47)	85(63)	0.009
< 2.0×10⁹/L, n. of patients (%)	93(53)	51(37)	
Median lymphocyte count, ×10⁹/L	1.600	2.016	0.006
Median neutrophil count, ×10⁹/L	0.218	0.200	NS
Median Hb level, g/dl	6.8	6.8	NS
Median reticulocyte count, ×10⁹/L	15.730	17.600	NS
Median platelet count, ×10⁹/L	10.000	11.000	NS
Interval from diagnosis to treatment, days	13	19	0.002

Multivariate variables	Odds ratio	95% CI	P
WBC count, < 2.0×10⁹/L	3.219	1.707-6.070	0.0003
Interval from diagnosis to treatment, < 30 days	2.571	1.225-5.396	0.012
Gender, male	1.873	1.042-3.366	0.036
Reticulocyte count, >25×10⁹/L	1.589	0.843-2.997	NS
Platelet count, >20×10⁹/L	1.362	0.657-2.826	NS
Etiology, hepatitis/others	1.223	0.504-2.966	NS

VSAA: very severe aplastic anemia; SAA, severe aplastic anemia; MAA, moderate aplastic anemia; WBC, white blood cell; Hb, hemoglobin.

count, that is, a relative increase in lymphocytes. Given the dramatic effects of T-cell suppressants including ATG and CyA on *in vivo* hematopoiesis, autoreactive T-cell responses against hematopoietic stem cells have been suggested to play a major role in the pathogenesis of AA, and *in vitro* studies have also supplied supportive evidence for this idea. Early experiments demonstrated inhibitory effects of autologous lymphocytes on hematopoietic progenitor cell growth through overproduction of cytokines such as interferon-γ and tumor necrosis factor-α by activated cytotoxic T cells in AA patients.[20-22] More recently, oligoclonal T-cell expansions have been described in AA patients, disappearing with clinical improvement following IST.[23] Taking our results and previous findings together, a higher WBC count before treatment may indicate the presence of numerous autoreactive T cells that need to be eliminated and thus a high potential to destroy marrow function through lymphocytes, rather than better residual marrow function. In this scenario, patients with a lower WBC count could be seen to have a better probability of hematopoietic recovery following IST.

We identified a significantly inverse correlation between response and interval between diagnosis and treatment; median intervals among responders and non-responders were 13 and 19 days, respectively (P=0.002). In categorical analysis, response rates of patients with intervals less than 30 and of 30 days or more were 60% and 43%, respectively (P=0.013). Figure 1C clearly indicates the inverse relationship. Notably, response rates to IST were considerably low among AA patients with long-standing disease; only 35%

of patients treated 90 days or more after diagnosis responded, suggesting that patients with this condition may receive irreversible damage to hematopoietic progenitor cells or stromal elements that progresses over time, possibly due to
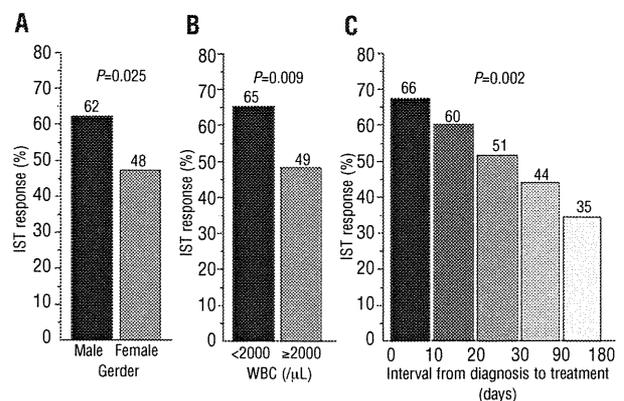

Figure 1. Response to IST in 312 patients according to WBC count, gender, and interval from diagnosis to treatment. (A) Response rate according to gender. Boys showed better response than girls (62% vs. 48%, respectively; P=0.025). (B) Response rate according to WBC counts. Patients with WBC count <2.0×10⁹/L displayed a significantly higher response rate than patients with WBC ≥2.0×10⁹/L (65% vs. 49%, respectively; P=0.009). (C) Response rate according to the interval between diagnosis and treatment. Response rate was inversely associated with the interval between diagnosis and treatment (P=0.002).

immune attack through autoreactivated lymphocytes. The present study indicates the importance of prompt IST therapy for patients with AA. We, therefore, recommend offering IST as soon as possible in all children with AA who lack a matched sibling donor.

Other variables did not differ significantly between responders and non-responders (Table 2). Particularly with regard to reticulocyte count, 122 patients showed reticulocyte count more than 25×10^9/L, of whom 67 (55%) responded to IST, and 186 patients had reticulocyte count of 25×10^9/L or less, of whom 107 (58%) responded to IST. Correlations of higher reticulocyte count and higher lymphocyte count at initial diagnosis with better response to IST in patients of all ages have recently been described by the National Institutes of Health (NIH) group.[15] However, when the same analysis was applied to their 77 pediatric patients, lymphocyte count was not predictive.[14] More recently, another relatively small study in adults with AA found no such association.[13] These studies were limited by inconsistency of regimens used for IST. The current study investigated a large cohort of children with AA treated using a unified regimen, but failed to confirm any correlation between reticulocyte count and response to IST, sug-

gesting a limited contribution of this clinical parameter to the prediction of hematopoietic recovery, at least in children.

In conclusion, pre-treatment clinical and laboratory findings influence response to IST. Favorable response correlates better with lower WBC count than with neutrophil count or disease severity, and this blood count parameter might help in clinically assessing bone marrow function. Unlike the situation in adult AA, reticulocyte count is not predictive of response to IST in pediatric patients. IST should be started as soon as possible after diagnosis of AA, given that the response rate worsens as the interval between diagnosis and treatment increases.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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- 69 -

MEETING REPORT

The Third Consensus Conference on the treatment of aplastic anemia

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Naoto Hirano · Jaroslaw Maciejewski · Joachim Deeg · Judith Marsh ·
Feng-Kui Zhang · Jong Wook Lee · Keiya Ozawa

1 Introduction

Acquired aplastic anemia (AA) is characterized by bone marrow hypoplasia and peripheral blood pancytopenia. Although the pathogenesis of AA is not well understood, it is thought to be an immune-mediated disease in most patients [1, 2]. The main treatment options for patients with AA include allogeneic bone marrow transplantation (BMT) and immunosuppressive therapy (IST). Recent studies of BMT from an HLA-matched family donor (MFD) showed excellent survival for AA patients. The long-term survival rate of children and young adults with severe aplastic anemia (SAA) after BMT from an MFD ranges from 70 to 90% [3, 4], and BMT currently represents first-line therapy if an MFD is available. The combination of antithymocyte globulin (ATG) and cyclosporine (CsA) results in a response rate of 60–70% in AA patients [5–7] and is indicated as first-line therapy in children and young adults if MFD is unavailable, as well as in all patients older than 40–50 years.

BMT from an HLA-matched unrelated donor (MUD BMT) is indicated for patients who have failed at least one course of ATG and CSA. Better HLA-typing and less-toxic preparative regimens have resulted in substantial increases in survival among patients undergoing MUD BMT [8–10].

Bearing these issues in mind, experts from Europe, America, and Asia presented recent advances in understanding of the pathophysiology and current clinical trials for the treatment of AA (Table 1) at the Third Consensus Conference on the treatment of aplastic anemia on February 21, 2010, in Hamamatsu, Japan. After all speakers had presented, a general consensus was held to establish guidelines for the diagnosis and treatment of AA. Participants included clinicians and scientists from 13 countries, including seven countries in Asia.

2 Pathogenesis of AA

In Session 1, four scientists presented the latest data regarding the pathogenesis of AA. Dr. Hirano identified two AA-associated antigens, kinectin and anti-postmeiotic segregation increased 1 (PMS1), by screening antibodies in a patient's sera against a peptide library of fetal liver cells [11]. The putative T cell epitope derived from kinectin triggered a cytotoxic T cell response in vitro, and inhibited granulocyte–macrophage colony forming unit formation. However, kinectin-specific T cells were not seen in AA patients. These auto-antibodies are present only in sera of AA patients, and become undetectable in the patients who achieve clinical remission, suggesting that these auto-antibodies may serve as a biomarker for AA, and may correlate with or predict disease activity in AA patients. However, a prospective study conducted by the Japan Childhood Aplastic Anemia Study Group failed to demonstrate a correlation between the presence of anti-PMS1 and response to IST [12].

Dr. Nakao discussed the clinical implication of detecting small paroxysmal nocturnal hemoglobinuria (PNH) clones by sensitive flow cytometric analysis. The presence of an increased number of PNH-type cells was predictive of a response to IST and a favorable prognosis among patients with AA. Ninety percent of patients with increased PNH-type cells responded to ATG + CSA, whereas only 50% of

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Springer

Table 1 Program

Session 1: S. Nakao, N. Young

Pathogenesis of aplastic anemia (AA)

(1) Autoimmunity in AA

   N. Hirano, Dana-Farber Cancer Institute, MA, USA

(2) Application of SNP-array in bone marrow failure syndromes

   J.P. Maciejewski, Cleveland Clinic Foundation, OH, USA

(3) PNH clones as a marker of autoimmunity

   S. Nakao, Kanazawa University Graduate School of Medicine, Japan

(4) Genetic risk factors for AA

   N. Young, National Heart, Lung, and Blood Institute, MD, USA

Session 2: S. Kojima, A. Bacigalupo

Stem cell transplantation

(1) Optimal conditioning regimen

   S. Kojima, Nagoya University Graduate School of Medicine, Japan

(2) Role of antithymocyte globulin

   A. Bacigalupo, Ospedale San Martino, Italy

(3) Long- term outcome after stem cell transplantation

   H.J. Deeg, Fred Hutchinson Cancer Research Center, WA, USA

Session 3: K. Ozawa, G. Socie

Immunosuppressive Therapy

(1) Optimal dose of rabbit-antithymocyte globulin

   J.C.W. Marsh, Kings College London, UK

(2) ATG + Cyclosporine vs High-dose Cyclophosphamide for treatment of aplastic anemia

   F.K. Zhang, Institute of Hematology & Blood Disease Hospital, China

(3) Role of G-CSF

   G. Socie, Hospital Saint Louis, France

(4) Role of iron chelator

   J.W. Lee, The Catholic University of Korea, Korea

Session 4: S. Nakao, S. Kojima, A. Bacigalupo

Discussion for General Consensus

patients without PNH-type cells responded. Failure-free survival rates were significantly higher among patients with minor PNH clones than among those without these cells [13].

A single nucleotide polymorphism array (SNP-array) has recently been applied widely as a powerful karyotyping tool that detects deletions, amplifications, and loss of heterozygosity (LOH) at high resolution [14]. Dr. Maciejewski used this new tool in a series of 102 AA patients. Using conventional metaphase cytogenetics, 13% of patients showed cytogenetic abnormalities, which increased to 26% when a SNP-array was used. Early detection of clonal lesions was also possible when using a SNP-array. Interestingly, loss of the short arm of chromosome 6, which

encompasses the HLA locus, was detected in three patients before IST. This finding suggests that escape from immune attack may work through the loss of the HLA haplotype in AA patients.

Telomeres are repeated nucleotide sequences that cap the ends of chromosomes and protect them from damage. Telomeres are short in one-third of AA patients [15]. Children with congenital bone marrow failure syndrome, and in particular, dyskeratosis congenita (DC), have extremely short telomeres [16]. Dr. Young demonstrated the presence of mutations in telomerase-complex genes such as TERT and TERC in a small percentage of AA patients without phenotypic characteristics of DC [17, 18]. A family study showed that healthy relatives of patients carrying these mutations also had short telomeres and mild hematologic abnormalities. Although telomere length does not predict response to IST, patients with short telomeres are at high risk of relapse and clonal evolution to myelodysplasia and acute myeloid leukemia after IST [19]. Dr. Young's group recently reported the significant correlation between absolute reticulocyte count (ARC) and absolute lymphocyte count at initial diagnosis and response to IST [20]. A further addition of telomere length increased the predictive capacity. Patients with both high ARC and long telomeres showed excellent survival, whereas those with low ARC and short telomeres had poor outcomes; patients with one of the two variables had intermediate outcomes.

3 Stem cell transplantation

In Session 2, three experts from Asia, Europe, and America discussed the optimization of stem cell transplantation for AA. Until the late 1990s, fewer than 40% of AA patients who underwent MUD BMT survived long-term, and there was a high incidence of graft failure and graft versus host disease (GVHD) [21]. Recent data have shown improved results through better selection of HLA-matched donors and changes in conditioning regimens [22, 23].

Dr. Kojima analyzed a Japanese cohort of 301 AA patients who received MUD BMT through the Japan Marrow Donor Program. Using matched-pair analysis, he showed the superiority of a fludarabine (Flu) + cyclophosphamide (CY) + ATG and radiation regimen compared with a CY + ATG + total body irradiation (TBI) regimen. The current recommended regimen in Japan includes Flu (100 mg/m^2) + CY (3,000 mg/m^2) + rabbit ATG (5 or 10 mg/kg) + 3 Gy TBI. He also used matched-pair analysis to compare tacrolimus (FK)/methotrexate (HTX) with CsA/MTX for the prophylaxis of GVHD in AA patients who received a MUD BMT. Results showed the superiority in overall survival of FK/MTX over CsA/MTX [24].

Dr. Bacigalupo proposed optimized protocols for BMT from an MFD for AA patients. For children and young adults, the recommended regimen is CY (200 mg/kg) + rabbit ATG (7.5 mg/kg). The stem cell source should be bone marrow rather than peripheral blood [25]. GVHD prophylaxis consists of CsA + MTX [26]. There is controversy concerning the upper age limit for BMT in AA patients. A large amount of data from the Europe Group for Blood and Marrow Transplantation (EBMT) showed an inferior outcome in AA patients older than 50 years, although outcomes for patients aged 30–40 years were similar to this aged 40–50 years. To improve outcome, Dr. Bacigalupo proposed a conditioning regimen with Flu (120 mg/kg), CY (1,200 mg/m^2), and rabbit ATG (7.5 mg/kg) for older patients. Dr. Bacigalupo also referred to previously published conditioning regimens for MUD BMT [22]. The current EBMT regimen recommended for children is Flu (120 mg/kg), CY (1,200 mg/m^2), and rabbit ATG (15 mg/kg). For adult patients, the addition of TBI (2 Gy) with a reduced dose of ATG (7.5 mg/kg) is recommended. However, a recent analysis of 100 patients treated according to these protocols revealed that graft failure and Epstein Barr virus (EBV)-lymphoproliferative disease (LPD) still remain significant causes of death [27]. Consequently, Dr. Bacigalupo modified the current EBMT protocol with an increased dose of CY (from 1,200 mg/m^2 to 120 mg/kg), a reduction of rabbit ATG (from 15 to 7.5 mg/kg), and prophylactic administration of rituximab for EBV-LPD.

In the United States, Dr. Deeg previously demonstrated an improved outcome in patients receiving CY + ATG + 2 Gy TBI for MUD BMT, compared with a higher dose of TBI [23]. The ongoing CTNN study in the United States is designed to find the best dose of CY (0, 50, 100, or 150 mg/kg) combined with a regimen of Flu, ATG, and 2 Gy TBI. The 0- and 150-mg trials stopped due to rejection and toxicities, respectively. Both regimens currently undergoing testing in Europe and the United States are similar to the regimen recommended by the Japanese group. Dr. Deeg discussed the late effects of stem cell transplantation and its major adverse effect, i.e., chronic GVHD. There are no benefits associated with chronic GVHD in patients with non-malignant diseases and it increases the risk of secondary malignancy [28]. The most significant risk factor for developing chronic GVHD is the use of peripheral blood stem cells [25]. Dr. Deeg recommended bone marrow, not peripheral blood, as the source of stem cells for AA patients. He analyzed risk factors for chronic GVHD in AA patients who received a matched-related BMT. Patients who received a nucleated marrow cell dose greater than 3.4 × 108/kg developed chronic GVHD 7.7 times more often than those who received a marrow cell dose less than 2.3 × 108/kg ($P = 0.004$). This

finding was further reflected in overall survival, which was significantly worse in patients who a received higher dose of bone marrow cells than in those who received a lower dose, although CD34 cell dose was not analyzed in this study.

4 Immunosuppressive therapy

Dr. Marsh summarized the clinical trials of IST with rabbit ATG and CsA for AA as the initial course of treatment [29, 30]. The dose of rabbit ATG varied between 10 and 18.75 mg/kg among studies. Response rates ranged from 50 to 70%, which was equivalent with rates seen with of horse ATG, although patient numbers reported from some of these studies were small. However, immunosuppression of rabbit ATG is more potent than horse ATG, resulting in an increased incidence of infectious complications [31]. Dr. Marsh concluded that it is warranted to conduct a prospective study to find the optimal dose of rabbit ATG, and that larger prospective studies comparing rabbit ATG with horse ATG are needed.

High-dose CY (HD-CY) without stem cell rescue has been developed as a promising therapy for AA by the John's Hopkin's group [32]. However, a randomized trial conducted by the National Institutes of Health showed unacceptable toxicities, leading to early closure [33]. Dr. Zhang compared HD-CY + CsA with ATG + CsA for treatment of AA. The dose of CY was decreased to 120 mg/kg from the original report of 200 mg/kg. The costs of drugs were much cheaper in the CY group than the ATG group in China. The response rate at 6 months was comparable between both groups at 70%. The overall survival at 3 years was also comparable between the two groups, at 85%. It is noteworthy that the rate of early death was less than 5% in the CY group. Dr. Zhang's data justify conducting a randomized study to compare a modified dose of CY therapy with standard ATG therapy for newly diagnosed.

5 AA patients

To date, three prospective randomized studies have addressed the role of granulocyte-colony stimulating factor (G-CSF) in combination with IST [6, 34, 35]. Dr. Socie summarized the results of these studies, which showed faster recovery of neutrophils in the G-CSF group but failed to show significant differences in study endpoints including response rate, incidence of infections, and overall survival between the G-CSF group and the non-G-CSF group. He also presented the latest EBMT study, which enrolled more than 200 newly diagnosed patients with AA [36]. The study also confirmed the results of previous

studies; there was no difference in overall survival or event-free survival between the two arms.

Dr. Lee discussed the role of iron chelation therapy in patients with AA. Regular transfusions lead to the development of iron overload, which is increasingly recognized as a risk factor following HSCT [37]. He presented the results of the EPIC trial, which evaluated the efficacy and safety of deferasirox, an oral iron chelator, in a large cohort of AA patients [38]. After 1 year of treatment, median serum ferritin levels decreased significantly with concomitant improvement of liver dysfunction. The therapy was generally well tolerated, but one quarter of patients suffered from an increase in serum creatinine levels. The concomitant use of CsA had a significant impact on serum creatinine levels.

6 Consensus panel

After all speakers had presented, a general consensus session was held. This session was chaired by S. Nakao, S. Kojima, and A. Bacigalupo. A number of questions were raised by the chairperson, and the following consensus was reached.

6.1 New diagnostic tests

The panelists discussed the relevance of incorporating new diagnostic tests into the management of AA patients. The new diagnostic tests include AA-associated autoantibodies, SNP-array, sensitive flow cytometric assay for PNH clones, and measurement of telomere length. All panelists felt that these new tests may be useful in the investigation of the pathophysiology of AA, but that it is too early to incorporate them into general practice for AA. The findings presented by each speaker must be confirmed by other investigators.

6.2 Stem cell transplantation

All panelists agreed that bone marrow should be used as the source of stem cells. The use of peripheral blood is indicated when a voluntary donor donates peripheral blood. A consensus was reached regarding the upper age limit both for BMT from an HLA-identical sibling and from an unrelated donor. The limit should be 50 years.

The chairperson proposed (1) CY + ATG for young patients and (2) CY + Flu + ATG for older patients as conditioning regimens in the case of HLA-matched sibling transplants. For adult patients transplanted from an unrelated donor, CY + Flu + ATG + low-dose TBI regimen was proposed. Although the panelists did not recommend other conditioning regimens, no general consensus was

reached on conditioning regimens. Results of ongoing CTNN study in the United States are expected to reveal the optimal conditioning regimen for unrelated BMT. A higher dose of stem cell infusion has been recommended to facilitate engraftment. According to the presentation by Dr. Deeg, however, a higher dose of stem cell infusion was harmful because of the associated increase with chronic GVHD. The panelists discussed the optimal dose of stem cells, but no agreement was reached. Dr. Deeg emphasized that all of the patients who receive HSCT for a rare disease such as AA should be enrolled into prospective studies to address unsolved questions. All panelists agreed that the donor should be matched at 10/10 or 9/10 levels by HLA high-resolution typing. In the case of patients in whom an appropriate donor is unavailable, unrelated cord blood transplantation or haploidentical transplantation may be indicated.

6.3 Immunosuppressive therapy

The combination of ATG and CsA remains the gold standard for immunosuppressive therapy. Because the supply of horse ATG was stopped in Europe and Asia, rabbit ATG replaced horse ATG in these areas. Because the optimal dose of rabbit ATG has not been clarified, a prospective study to compare two doses of rabbit ATG is proposed. In addition, the panelists discussed the rationale of performing a randomized study to compare a modified dose of high-dose CY + CsA with ATG + CsA as first-line therapy.

Although several panelists agreed with the need for such a study, the majority of the panelists did not place a high priority on this type of study. Most panelists thought that G-CSF is indicated only in limited cases, for example, patients with severe bacterial or fungal infections. Although all published randomized studies revealed that G-CSF has no proven effect on clonal evolution in AA, several panelists felt that a longer follow-up period is necessary to reach a definitive conclusion on this issue.

Appendix

The following persons participated in the conference

Hoon Kook (Gwangiu, Korea), Dae Chul Jeong (Seoul, Korea), Jong Wook Lee (Seoul, Korea), Surapol Issaragagrisil (Bankok,Thailand), Xiao-Fan Zhu (Tianjin, China), Feng-Kui Zhang (Tianjin, China), Jing-Yan Tang (Shanghai, China), Jianping Shen (Hangzhou, China), Minghui Duan (Beijing, China), Jun Ma (Harbin, China), Honorata Baylon (Manila, Philippine), See Voon Seow (Singapore), Michelle Poon (Singapore), Lily Wong Lee (Sabah, Malaysia), Naoto Hirano (Boston, USA), Jaroslaw Maciejewski (Cleveland, USA), Neal Young (Bethesda, USA),

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Joachim Deeg (Seattle, USA), Andrea Bacigalupo (Geno-va, Italy), Judith Marsh (London, UK), Gerard Socie (Paris, France), Keiya Ozawa (Tochigi, Japan), Masao Tomonaga (Nagasaki, Japan), Shinji Nakao (Kanazawa, Japan), Hiroto Yamazaki (Kanazawa, Japan), Akio Urabe (Tokyo, Japan), Seishi Ogawa (Tokyo, Japan), Hiroki Yamaguchi (Tokyo, Japan), Masanao Teramura (Tokyo, Japan), Kensuke Usuki (Tokyo, Japan), Chitose Ogawa (Tokyo, Japan), Ohara Akira (Tokyo, Japan), Tatsutoshi Nakahata (Kyoto, Japan), Hiromasa Yabe (Isehara, Japan), Etsuro Ito (Hirosaki, Japan), Kazuko Kudo (Shizuoka, Japan), Seiji Kojima (Nagoya, Japan), Yoshiyuki Takahashi (Nagoya, Japan), Haruhiko Ohashi (Nagoya, Japan), Koichi Miyamura (Nagoya, Japan).

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