In our previous study, AA patients carrying DRB1*1502, another major allele corresponding to HLA-DR15 in Japanese, did not show a better response to CsA than those without HLA-DR15 [6]. The exact role of HLA-DR15 in the immune mechanisms of AA thus remains unclear, probably because of the low number of patients that have been studied for DRB1 alleles and the general heterogeneity in the pathogenesis of AA.

Another interesting aspect of HLA-DR15 is the association with the expansion of paroxysmal nocturnal hemoglobinuria (PNH) clones. Several studies have revealed the frequency of HLA-DR15 to be significantly higher in patients with AA and MDS possessing PNH-type blood cells and in florid PNH than in normal controls [10,12], however, the relationship between DRB1 alleles corresponding to DR15 and increased PNH-type cells in AA has not yet been studied in detail. The close relationship between HLA-DR15 and the expansion of PNH clones suggests that the T-cell responses against certain antigen presented by HLA-DR15 or other HLA-class II alleles in linkage disequilibrium with DR15 in hematopoietic stem cells may cause bone marrow failure, thus allowing PNH-type stem cells to survive.

We previous demonstrated the frequency of HLA-DR15 to markedly increase in patients with MDS-refractory anemia (RA) and a small population of PNH-type cells ($>0.003\%$ for granulocyte, $>0.005\%$ for red blood cells [RBCs]), as demonstrated by sensitive flow cytometry [13]. In that study, RA patients possessing a small population of PNH-type cells displayed favorable responses to CsA. An investigation of a large number of AA patients treated with IST using the same methods to detect small populations of PNH-type cells would thus clarify the role of DRB1 alleles corresponding to HLA-DR15 and PNH-type cells in the immune mechanisms of AA and their mutual relationships. To test this hypothesis, we investigated the relationship between the DRB1 allele in such patients and both the presence of a small population of PNH-type cells and the response to ATG plus CsA therapy in 140 Japanese AA patients.

Materials and methods

Patients

Table 1 summarizes the patient characteristics. The 140 Japanese AA patients were diagnosed at Kanazawa University Hospital, hospitals that participate in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions from April 1999 through November 2005. The study subject included 77 patients who were tested for any correlation between the presence of a minor population in PNH-type cells and the response to IST in our previous study [14]. The severity of AA was classified according to the criteria proposed by Camitta [15] and Marsh et al. [16]. All participants provided written, informed consent to all procedures associated with the study, which was approved by the Ethical Committee at our institution (study number 46). This study also conforms to the recently revised tenets of the Helsinki protocol.

Table 1. Patient characteristics

Characteristics	n	Range
Total (n)	140	NA
Age at diagnosis (y)	60	12–92
Gender: Male/female	65/75	NA
Severity: Severe/moderate	65/75	NA
Neutrophil count ($\times 10^9$/L)	720	0–2226
Platelet count ($\times 10^9$/L)	20	2–118
Reticulocyte count ($\times 10^9$/L)	28	2–106
No. of patients with clonal abnormality (n)	11	NA

NA = not applicable.

Detection of PNH-type cells

We performed two-color flow cytometry of the granulocytes and RBCs according to our previously described method [14,17,18]. First, 3–5 mL heparinized blood was drawn from each patient. To detect the PNH-type granulocytes, phycoerythrin (PE)-labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Mountain View, CA, USA), fluorescein-isothiocyanate (FITC)-labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA, USA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) were used in combination with isotype-matched control mAbs, as described previously. To detect PNH-type RBCs, PE-labeled anti-glycophorin A mAbs (clone JC159, DAKO, Glostrup, Denmark) were used instead of anti-CD11b mAbs . Fresh blood was diluted to 3% using phosphate-buffered saline, and 50 mL diluted blood was incubated with 4 mL PE-labeled anti-glycophorin A mAbs , FITC-labeled anti-CD55 and anti-CD59 mAbs on ice for 25 minutes. A total of at least 1×10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using FACScan flow cytometry (Becton Dickinson). In order to avoid any false-positive results, we excluded $CD11b^{dim}$ and glycophorin A^{dim} cells from the analyses using careful gating because these cells include damaged cells those are often mistakenly judged to be PNH-type cells because of their poor binding to anti-CD55 and anti-CD59 mAbs. This flow cytometry method failed to detect 0.003% or more $CD55^-CD59^-CD11b^+$ granulocytes or 0.005% or more $CD55^-CD59^-$glycophorin-A^+ RBCs in any of 183 healthy individuals. We, therefore, defined the presence of $>0.003\%$ $CD55^-CD59^-CD11b^+$ granulocytes $CD55^-CD59^-$glycophorin-A^+ RBCs to be abnormal [14,18].

Determination of DRB1 alleles

DRB1 alleles of 140 AA patients and 491 healthy Japanese randomly selected from general population [19] were determined using polymerase chain reactions with sequence-specific primers (PCR-SSP) (Micro SSP HLA DNA typing trays; One Lambda, Canoga Park, CA, USA). Genomic DNA was prepared from blood samples using a DNA extraction kit (Generation capture column kit; Gentra, Minneapolis, MN, USA).

HSV-pDCs and IL-3–pDCs induce granzymes and perforin in CD4+ T cells in a partially IL-10–dependent manner

It has recently been shown that anti-CD3/anti-CD46 stimulation induces CD4+ regulatory T cells that express granzymes and perforin.[21,22] Because perforin has been shown to play an important role in down-regulating T-cell responses in chronic viral infection,[20] we examined whether HSV-pDCs induce the expression of granzymes and perforin in naive CD4+ T cells. We cocultured allogeneic naive CD4+ T cells with HSV-pDCs, IL-3–pDCs, or GM-CSF–mDCs for 8 days in the presence or absence of anti-IFN-α/β receptor mAb or anti-IL-10 mAb, and examined the expression of granzyme A, granzyme B, and perforin by intracellular staining. Unstimulated naive CD4+ T cells did not express detectable levels of granzyme A, granzyme B, or perforin (data not shown). As shown in Figure 5, granzyme A was induced by stimulation with any DCs: HSV-pDCs, IL-3–pDCs, and GM-CSF–mDCs. HSV-pDCs, and to a lesser extent IL-3–pDCs, induced high levels of granzyme B in CD4+ T cells, whereas GM-CSF–mDCs induced only a low level of granzyme B. HSV-pDCs, and to a lesser extent IL-3–pDCs, induced moderate levels of perforin, whereas GM-CSF–mDCs did not induce a significant level of perforin. The induction of granzyme A or granzyme B by HSV-pDCs did not diminish in the presence of anti-IFN-α/β receptor mAb, whereas the induction of perforin moderately diminished. The induction of the 3 molecules by HSV-pDCs or IL-3–pDCs partially but substantially diminished in the presence of anti-IL-10 mAb. These data indicate that HSV-pDCs and to a lesser extent IL-3–pDCs, but not GM-CSF–mDCs, induce CD4+ T cells to express granzyme B and perforin in a partially IL-10–dependent manner. Type I IFNs may also be involved in the induction of perforin.

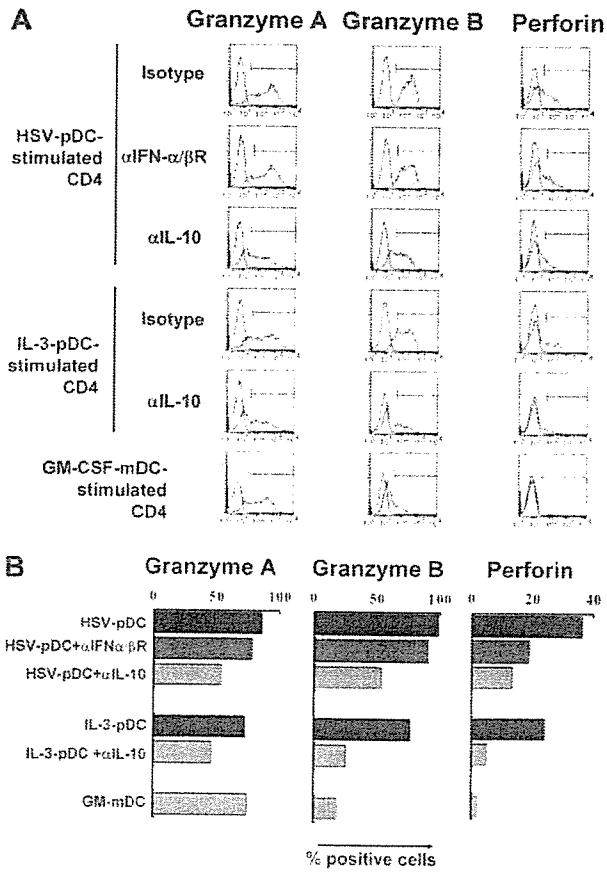

Figure 5. HSV-pDCs and IL-3–pDCs induce granzymes and perforin in CD4+ T cells in a partially IL-10–dependent manner. Naive CD4+ T cells were stimulated with allogeneic HSV-pDCs or IL-3–pDCs for 8 days in the presence of isotype-matched control mAb, anti-IFN-α/β receptor mAb, or anti-IL-10 mAb. Alternatively, T cells were stimulated with allogeneic GM-CSF–mDCs for comparison. Activated T cells were gated based on the expression of CD25, and expression of intracellular granzyme A, granzyme B, and perforin was analyzed by flow cytometry. (A) Histograms. Open histograms represent cells stained with isotype-matched control mAbs. (B) Percentages of cells expressing the 3 molecules, indicated with markers in panel A. The data shown are representative of 3 experiments.

CD4+ T cells stimulated with HSV-pDCs exhibit perforin-dependent cytotoxicity

Finally, we examined whether CD4+ T cells stimulated with the 3 types of DCs have cytotoxic activity in accordance with the expression of granzymes and perforin. We used a Burkitt lymphoma cell line Daudi or a myelomonocytic cell line U937 as target cells of cytotoxicity assay. We stained the target cells with CFSE[22] and cocultured the effector and target cells for 4 hours. Then the percentages of killed CFSE-labeled target cells were calculated by staining them with 7-AAD. As shown in Figure 6A, a large proportion of Daudi cocultured with HSV-pDC–stimulated CD4+ T cells underwent apoptosis. Daudi cocultured with IL-3/pDC-stimulated CD4+ T cells also underwent apoptosis, albeit to a lesser extent. The extent of apoptosis in both conditions was correlated with E/T ratios. In contrast, Daudi cocultured with GM-CSF/mDC-stimulated CD4+ T cells minimally underwent apoptosis. The apoptosis induced by HSV-pDC–stimulated CD4+ T cells was substantially diminished by the addition of EGTA, indicating that the death was perforin-dependent (Figure 6B). The apoptosis induced by IL-3-pDC–stimulated CD4+ T cells was marginally inhibited by EGTA. We obtained similar results using U937 as target cells (data not shown). As target cells, we also used activated T cells from the same donors as DC donors (allospecific targets), or activated T cells from donors different from DC and effector T-cell donors (third-party targets). Third-party target T cells (Figure 6C) as well as allospecific target T cells (data not shown) were substantially killed by HSV-pDC–stimulated CD4+ T cells and were also killed by IL-3–pDC–stimulated CD4+ T cells to a lesser extent, whereas the killing by GM-CSF–mDC–stimulated CD4+ T cells was minimal, as observed in the killing of unrelated tumor cell lines. These data indicate that in proportion to the degree of up-regulation of granzymes and perforin, CD4+ T cells stimulated with HSV-pDCs have the strongest perforin-dependent cytotoxic activity among the 3 CD4+ T cells stimulated with different DCs in an antigen-nonspecific manner. CD4+ T cells stimulated with IL-3–pDCs may also have a significant cytotoxicity.

Discussion

During immune responses to microbial pathogens, overwhelming pathologic immune reactions need to be avoided to minimize tissue damage. IFN-γ/IL-10–producing CD4+ T cells have been recognized in infections by several pathogens,[2,4-6] and may play an important role in down-modulating pathologic immune reactions and may also be responsible for allowing persistent infection.[2,3,7,29] Given that the direction of naive CD4+ T-cell differentiation is largely determined by the subset and activation state of DCs, it is important to elucidate which type of DCs induces naive CD4+ T cells to differentiate into immunoregulatory IFN-γ/IL-10–producing T cells during infection. Here we showed that the IFN-γ/IL-10–producing CD4+ T cells induced by HSV-pDCs

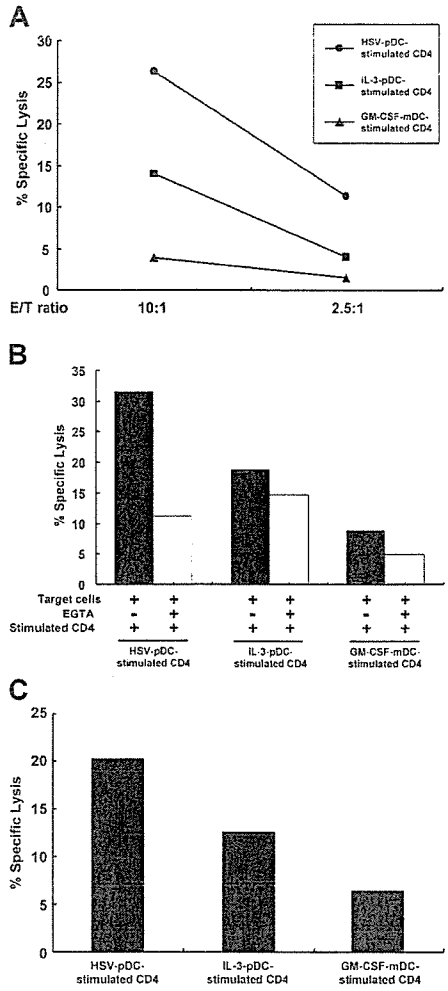

Figure 6. CD4⁺ T cells stimulated with HSV-pDCs exhibit perforin-dependent cytotoxicity. (A) Naive CD4⁺ T cells stimulated with the 3 types of allogeneic DCs for 8 days (effector cells) were cocultured with CFSE-labeled Daudi target cells at an E/T ratio of 10:1 or 2.5:1 for 4 hours. Immediately before analysis, 7-AAD was added to each sample, and percentages of 7-AAD⁺ cells were analyzed by flow cytometry. Basal lysis of the target cells was 9.6%. The data shown are representative of 2 experiments. (B) Naive CD4⁺ T cells stimulated with the 3 types of allogeneic DCs for 8 days (effector cells) were cocultured with CFSE-labeled Daudi target cells at an E/T ratio of 30:1 for 4 hours in the absence or presence of EGTA. Percentages of 7-AAD⁺ cells were analyzed by flow cytometry as shown in panel A. Basal lysis of the target cells was 18%. Filled and open bars represent percent specific lysis induced in the absence or presence of EGTA, respectively. The data shown are representative of 3 experiments. (C) Naive CD4⁺ T cells stimulated with the 3 types of allogeneic DCs for 8 days (effector cells) were cocultured with CFSE-labeled activated T cells from third-party donors (target cells) at an E/T ratio of 30:1 for 4 hours. Percentages of 7-AAD⁺ cells were analyzed by flow cytometry as shown in panel A. Basal lysis of the target cells was 16%. The data shown are representative of 2 experiments.

acquire anergic and regulatory properties by the action of pDC-derived type I IFNs and T cell–derived IL-10. Interestingly, cytotoxic molecules perforin and granzyme B in CD4⁺ T cells are induced by pDCs in an IL-10–dependent manner. These results suggest an immunoregulatory role of type I IFN-producing pDCs through the induction of perforin/granzyme-expressing regulatory CD4⁺ T cells and present a possible mechanism by which pDCs induce coordinated antiviral immunity by preventing excessive immune responses.

Several studies have shown that pDCs are capable of inducing immunoregulatory T cells through various mechanisms. For example, pDC precursors induce CD4⁺ T-cell anergy, apparently due to the lack of costimulatory molecules.[30] IL-3/CD40L-stimulated pDCs induce IL-10–producing CD8⁺ regulatory T cells.[31] pDCs stimulated with CpG ODNs induce Foxp3-expressing CD4⁺CD25⁺ regulatory T cells.[32] Mouse in vivo studies have shown that lung pDCs prevent asthmatic reactions to harmless inhaled antigens by inducing regulatory T cells.[33] pDC precursors contained in the graft facilitate allogeneic hematopoietic stem cell engraftment.[34] These findings indicate that pDC precursors that express low levels of costimulatory molecules or pDCs activated with particular stimulations are capable of inducing anergic or regulatory T cells. However, these studies do not ascribe the induction of T-cell suppression to type I IFNs. Here we show the importance of type I IFNs in inducing anergic and regulatory T cells by pDCs under the conditions where viral stimuli induce them to produce the cytokines. Although murine pDCs have been shown to induce anergic T cells through the expression of indoleamine 2,3-dioxygenase (IDO),[35,36] the addition of an IDO inhibitor 1-methyl-D-tryptophan to the coculture of pDCs and naive CD4⁺ T cells did not inhibit the induction of regulatory T cells (unpublished data, September 2004), indicating that IDO is not involved in the system shown in this study.

Recent studies have been revealing an immunoregulatory role of type I IFNs through different mechanisms. First, IFN-α treatment of naive CD4⁺ T cells delays their entry into cell cycle early on T-cell receptor (TCR) triggering and sensitizes T cells to activation-induced cell death later after activation.[37,38] These mechanisms may explain the markedly suppressed proliferation of naive CD4⁺ T cells stimulated with type I IFN-producing HSV-pDCs. The attenuated proliferation of CD4⁺ T cells stimulated with virus-activated pDCs may result in accumulation of IFN-γ/IL-10–producing regulatory CD4⁺ T cells at a chronic stage during viral infection.

Second, type I IFNs have been shown to induce CD4⁺ T cells that produce IL-10 together with IFN-γ. For example, polyclonal stimulation of human naive CD4⁺ T cells using anti-CD3 mAb in the presence of IFN-α induce IFN-γ/IL-10–producing CD4⁺ T cells that have regulatory activity.[18,39] Type I IFN receptor-deficient mice generate reduced numbers of IL-10–producing CD4⁺ T cells, which correlates with enhancement of CD8⁺ T-cell responses, suggesting that type I IFNs suppress CD8⁺ T-cell responses through the induction of IL-10–producing CD4⁺ regulatory T cells.[19] The present study suggests that type I IFN-producing pDCs may be the APCs responsible for the induction of type I IFN-dependent CD4⁺ regulatory T cells.

IFN-γ/IL-10–producing CD4⁺ T cells are identified in hosts suffering from chronic or persistent infections with various types of pathogens.[2,4-6] Such T cells have been suggested to be responsible for permitting persistent infection.[2] In addition, IL-10–producing CD4⁺ regulatory T cells generated in mice with retroviral infection have been shown to contribute to viral persistence.[40] These IL-10–producing T cells may also play an important role in preventing excessive immune responses, as the absence of IL-10 leads to uncontrolled lethal immune responses.[1] An important question is what types of DCs induce immunoregulatory IFN-γ/IL-10–producing CD4⁺ T cells frequently identified in infected hosts. In mice, it has been shown that IL-12/IL-10–producing CD8α⁺ DCs stimulated with heat-killed *Listeria* induce IFN-γ⁻IL-10⁺ regulatory CD4⁺ T cells that inhibit airway hyperreactivity.[41] IL-12 has been shown to induce human CD4⁺ T cells to produce IL-10 together with IFN-γ.[5,42] In humans, mDCs produce IL-12 but not IFN-α in response to bacterial stimuli, whereas pDCs produce IFN-α but not IL-12 in response to viral stimuli.[10,15] Therefore, in humans, IL-12–producing mDCs may induce immunoregulatory

ATG plus CsA therapy and response criteria

Seventy-seven of 140 patients (55.0%) were treated with ATG (15 mg/kg/day, 5 days; Lymphoglobuline, Aventis Behring, King of Prussia, PA, USA) and CsA (Novartis, Basel, Switzerland, 6 mg/kg/day) within 1 year of diagnosis. The dose of CsA was adjusted to maintain trough levels at between 150 and 250 ng/mL and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (filgrastim, 300 $\mu g/m^2$ or lenograstim, 5 $\mu g/kg$) was administered to some patients. The response to ATG plus CsA therapy was evaluated according to the response criteria described by Camitta [20]. A complete response was defined as hemoglobin normal for age, neutrophil count $>1.5 \times 10^9/L$, and platelet count more than $150 \times 10^9/L$. A partial response was defined as transfusion-independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling of the normalization of at least one cell line or an increase in the baseline hemoglobin of more than 30 g/L (if initially <60 g/L), a neutrophil count of $>0.5 \times 10^9/L$ (if initially $<0.5 \times 10^9/L$), and a platelet count of more than $10 \times 10^9/L$ (if initially $<20 \times 10^9/L$) in patients with moderate AA.

Statistical analysis

The allele frequency defined as the proportion of patients with at least one copy of a specific gene was determined by direct counting. The χ^2 test compared the allele frequencies of HLA-DRB1 between the patient groups and a Japanese control population, composed of 491 healthy unrelated individuals selected at random from the general population [19]. The corrected value of p (p_c) was calculated by multiplying p with the number of alleles tested (n = 30). The χ^2 test, Fisher's exact test, and logistic procedures [21] analyzed associations between prevalence of increased PNH-type cells and genetic factors, and between individual pretreatment variables and the response to ATG plus CsA therapy. The Kaplan-Meier methods graphically compared the cumulative incidence of the response to ATG and CsA therapy and the time to event, while the log-rank test analyzed differences between the patients who possess HLA-DRB1*1501, DRB1*1502 and DRB1 alleles other than these two alleles. All statistical analyses were performed using the JMP version 5.0.1J software program (SAS Institute, Cary, NC, USA).

Table 2. Frequencies of HLA-DRB1 alleles in Japanese AA patients and controls

	AA patients (n = 140)		Controls (n = 491)		
HLA-DRB1 allele	n	%*	n	%*	p_c value**
0101	10	7.1	64	13.0	NS
0301	0	0.0	4	0.8	NS
0401	2	1.4	17	3.5	NS
0403	4	2.9	18	3.7	NS
0404	0	0.0	2	0.4	NS
0405	35	25.0	129	26.3	NS
0406	5	3.6	32	6.5	NS
0407	2	1.4	2	0.4	NS
0409	0	0.0	1	0.2	NS
0410	1	0.7	17	3.5	NS
0701	0	0.0	2	0.4	NS
0801	0	0.0	0	0.0	NS
0802	6	4.3	36	7.3	NS
0803	8	5.7	84	17.1	NS
0901	36	25.7	148	30.1	NS
1001	2	1.4	2	0.4	NS
1101	7	5.0	22	4.5	NS
1201	7	5.0	34	6.9	NS
1202	2	1.4	12	2.4	NS
1301	0	0.0	4	0.8	NS
1302	11	7.9	61	12.4	NS
1401	2	1.4	21	4.3	NS
1402	0	0.0	2	0.4	NS
1403	4	2.9	13	2.6	NS
1405	4	2.9	18	3.7	NS
1406	2	1.4	10	2.0	NS
1407	0	0.0	1	0.2	NS
1501	47	33.6	63	12.8	<0.01
1502	61	43.6	120	24.4	<0.01
1602	2	1.4	4	0.8	NS

AA = aplastic anemia; NS = not significant.
*Allele frequencies were determined by dividing the number of patients carrying one or two specific alleles by the total number of individuals.
**Corrected p value (p_c) was calculated by multiplying the p value with the number of alleles (n = 30) tested.

Results

Frequencies of DRB1 alleles in AA patients

Table 2 summarizes the frequencies for the 30 different DRB1 alleles identified in the 140 AA patients and 491 controls. Only the frequencies of DRB1*1501 (33.6% vs 12.8%, $p_c < 0.01$, odds ratio = 3.43) and DRB1*1502 (43.6% vs 24.4%, $p_c < 0.01$, odds ratio = 2.39) were significantly higher among the AA patients than among controls. Figure 1 illustrates the numbers of patients with DRB1*1501 and/or DRB1*1502 and the patients without either of the two alleles in the different age groups. Two peaks in the age distribution of the patients were noted, namely, at 20 to 29 years old and at 60 to 79 years old. After dividing the patients into young (younger than 40 years old, n = 37) and old (40 years or older, n = 103) groups, 82.5% of patients in the older group carried at least one of DRB1*1501 or DRB1*1502. Frequency of DRB1*1502 in the older group (54 of 103 patients, 52.4%) was significantly higher ($p_c = 0.03$) than that in the younger group (6 of 37 patients, 16.2%). No significant difference in the frequency of DRB1*1501 was identified between the two groups (36 of 103 patients, 35.0% vs 11 of 37 patients, 29.7%, $p = 0.56$).

Prevalence of patients possessing PNH-type cells

A wide range of PNH-type granulocytes (0.005–23.0%; median, 0.153%) and PNH-type RBCs (0.007–6.57%; median, 0.094%) were detected in 92 of 140 (65.7%) AA patients. When patients were divided into four groups according to presence of DRB1*1501 and DRB1*1502, the proportions of PNH^+ patients were 66.7% (4 of 6 patients) in the

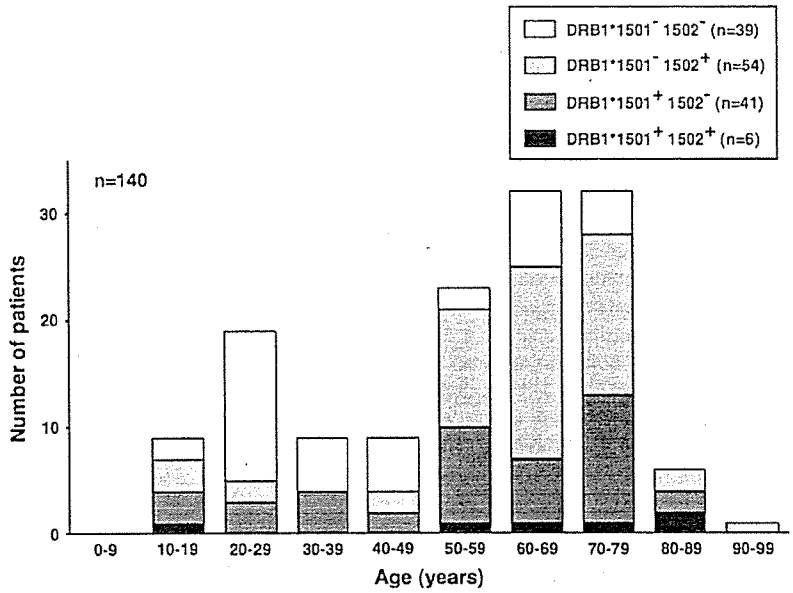

Figure 1. Age distribution of aplastic anemia (AA) patients with or without HLA-DR15. The number of AA patients with or without HLA-DR15 in different age groups is shown. DRB1*1501$^+$1502$^+$, patients with both DRB1*1501 and DRB1*1502; DRB1*1501$^+$1502$^-$, patients with DRB1*1501 but not DRB1*1502; DRB1*1501$^-$1502$^+$, patients with DRB1*1502 but not DRB1*1501; DRB1*1501$^-$1502$^-$, patients with neither DRB1*1501 nor DRB1*1502.

DRB1*1501$^+$1502$^+$ patients, 85.3% (35 of 41 patients) in DRB1*1501$^+$1502$^-$, 59.3% (32 of 54 patients) in DRB1*1501$^-$1502$^+$ and 53.8% (21 of 39 patients) in DRB1*1501$^-$1502$^-$.

Allele frequencies in the PNH$^+$ and PNH$^-$ AA patients

We next divided the 140 AA patients for whom both DRB1 alleles were determined into PNH$^+$ patients (n = 92) and patients without a small population of PNH-type cells (PNH$^-$ patients, n = 48), and then compared the frequency of each DRB1 allele among the three different groups including the PNH$^+$ patients, PNH$^-$ patients, and controls (Fig. 2). The frequency of DRB1*1501 compared to the controls was significantly higher in only the PNH$^+$ patients (39 of 92 patients, 42.4%, $p_c < 0.01$), not in PNH$^-$ patients (8 of 48 patients, 16.7%). On the other hand, the frequency of DRB1*1502 in comparison to the controls was higher in both the PNH$^+$ patients (37 of 92 patients, 40.2%, $p_c = 0.05$) and PNH$^-$ patients (24 of 48 patients, 50.0%). Frequencies of other DRB1 alleles, including DRB1*0405, were similar among PNH$^+$ patients, PNH$^-$ patients, and controls.

Correlation of HLA-DR15 alleles with the prevalence of increased PNH-type cells in AA patients

We analyzed the associations between the prevalence of PNH-type cells and genetic factors, such as age, sex, severity, chromosomal abnormality, and HLA-DRB1 allele to determine which factors might contribute to a slight increase in PNH-type cells in our AA patients. The presence of DRB1*1501 ($p < 0.01$, odds ratio = 3.68) was the only significant factor associated with an increase in the proportion of PNH-type cells based on a univariate analysis, and a multivariate analysis confirmed this result ($p < 0.01$). The presence of DRB1*1502 was not considered to be a contributing factor.

Favorable factors affecting response to ATG plus CsA therapy

Fifty-five of 77 patients (71.4%) improved with ATG plus CsA therapy. The factors favorably affecting the response to IST in the AA patients were examined under a univariate and multivariate analysis (Table 3). Only the presence of PNH-type cells was significantly associated with the response to IST based on a multivariate analysis. After taking into account the kinetics of the response to treatment, we made Kaplan-Meier curves to determine the probability of response to IST in three different groups of patients as defined by DRB1 alleles (Fig. 3). There were significant differences in the probability of the response to IST between the DRB1*1501$^+$1502$^-$ patients and either the DRB1*1501$^-$1502$^+$ patients ($p < 0.01$) or the DR15$^-$ patients ($p = 0.01$) (Fig. 3A). However, these differences in the probability of response IST were no longer observed when the probability of response was compared in either the PNH$^+$ patients or the PNH$^-$ patients (Fig. 3B, C).

Discussion

This study demonstrated for the first time that, in addition to DRB1*1501, which is a major DRB1 allele determining

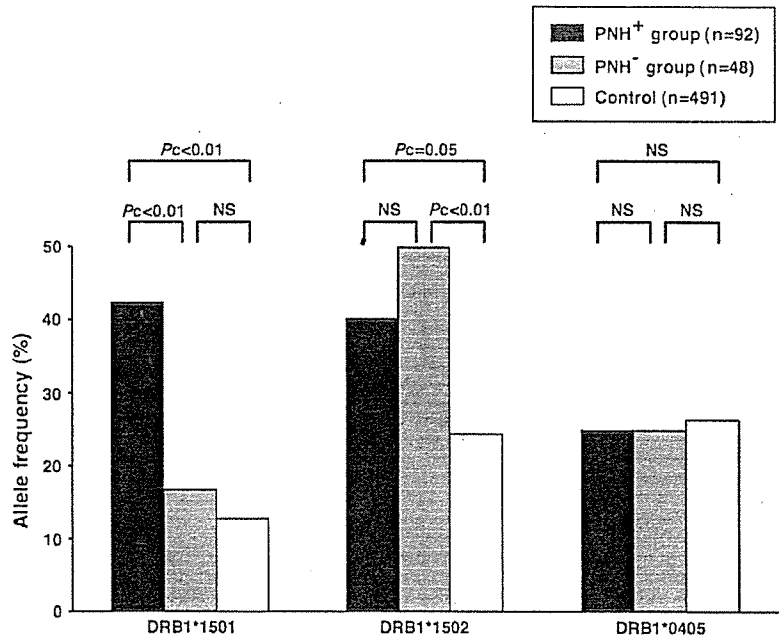

Figure 2. HLA-DRB1 allele frequencies in paroxysmal nocturnal hemoglobinuria $(PNH)^+$ and PNH^- aplastic anemia (AA) patients. Frequencies of the three alleles, DRB1*1501, DRB1*1502, and DRB1*0405 are compared in the PNH^+ AA patients, PNH^- AA patients, and controls.

the presentation of HLA-DR15 in Caucasian [2,3] and Chinese populations [4], DRB1*1502 is frequently present in Japanese AA patients. This finding, based on a large number of patients, suggests that the DR15 molecule plays a definite role in development of a subset of AA. Another novel finding in the present study was that the significantly increased frequency of HLA-DR15 was only observed in old AA patients. The frequency of HLA-DR15 reached up to 80% in AA patients 40 years of age or older. The apparent age-dependent differences in HLA-DR15 frequency suggest that the pathophysiology of AA in older patients may therefore differ from that in younger patients. Several studies of Japanese pediatric patients have revealed a relatively high incidence of MDS secondary to AA compared to adult patients [22–24]. Given the lower frequency of HLA-DR15, pediatric AA may thus display a higher proportion of bone marrow failure caused by nonimmune mechanisms than adult AA.

In contrast to the findings of previous reports, DRB1*1501 appeared to confer a better chance of response to regimens including ATG than other DRB1 alleles, including DRB1*1502. We previously demonstrated that DRB1*1501 predicts the response to CsA, but not to ATG [11]. In the previous study, only 6 of 59 ATG-treated patients received CsA. The combined use of CsA and the larger number of ATG-treated patients in the present study probably accounts for the different findings regarding the role of DRB1*1501 in predicting the response to ATG therapy. DRB1*1501 may affect the response of AA to ATG therapy only when CsA is administered in combination with ATG.

Several previous studies failed to confirm the role of HLA-DR15 in predicting the response to ATG [3,10]. Most previous studies analyzed DRB1 alleles using low-resolution methods that are unable to sufficiently distinguish DRB1*1502 from DRB1*1501. DRB1*1502 accounts for 3% to 7% of the DRB1 alleles corresponding to DR15 even in Caucasians [25], and this frequency may even be higher in AA patients, particularly among AA patients 40 years of age or older. As a result, some patients with DR15 who did not respond to ATG in previous studies may have been $DRB1*1502^+$, rather than $DRB1*1501^+$. The results of this study indicate the importance of

Table 3. Pretreatment variables associated with a response to antithymocyte globulin plus cyclosporin A therapy

	p Value	
Favorable factors	univariate*	multivariate**
Gender (male vs female)	0.32	0.47
Age (at least 40 y vs younger)	0.79	0.37
Severity (severe vs moderate)	0.61	0.86
HLA-DRB1*1501 (positive vs negative)	0.03	0.19
HLA-DRB1*1502 (positive vs negative)	0.61	0.46
PNH-type cells (positive vs negative)	<0.01	<0.01

*Fisher's exact probability test.
**Wald c^2 test for a logistic regression model.
PNH = paroxysmal nocturnal hemoglobinuria.

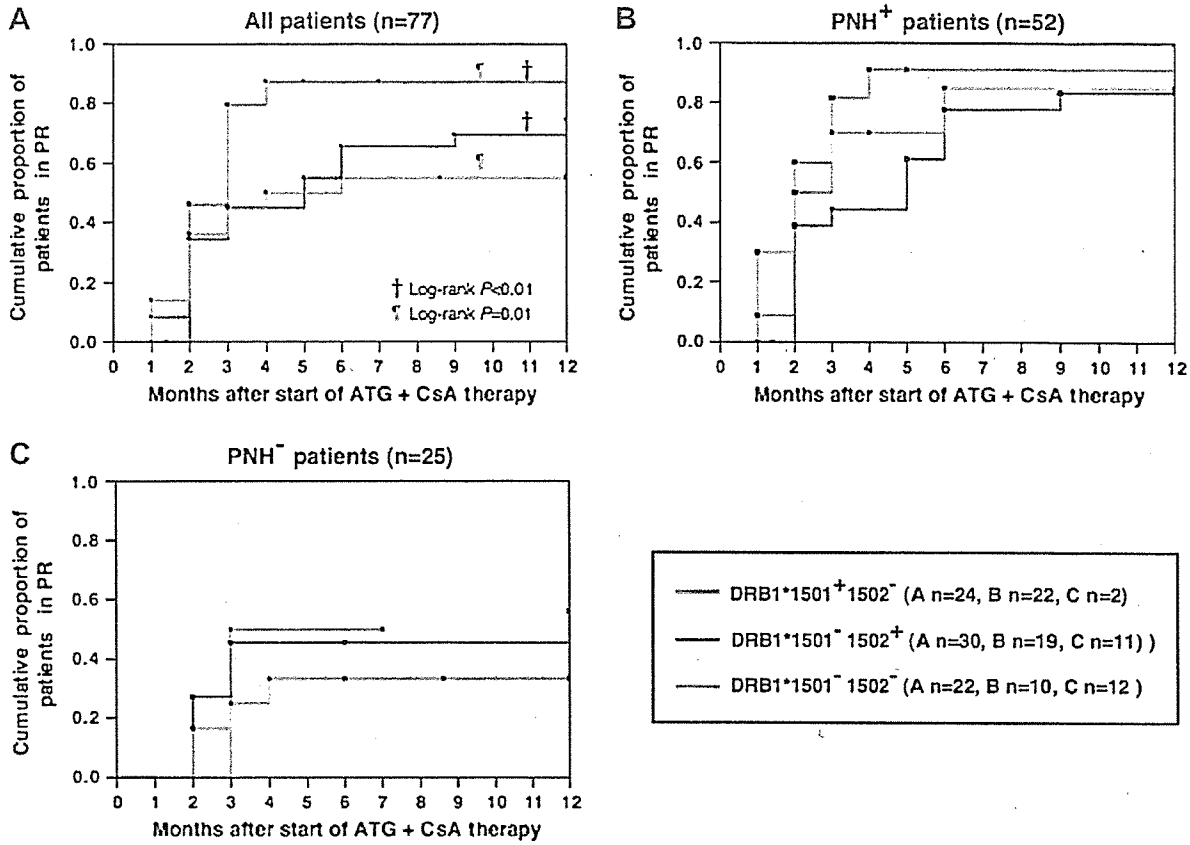

Figure 3. Kinetics of response to antithymocyte globulin (ATG) plus cyclosporin A (CsA) therapy. Kaplan-Meier curves for the response in the different groups of patients based on the DRB1 alleles are shown. DRB1*1501^{+}1502^{+} patients were not showed in this figure because only one patient (he was paroxysmal nocturnal hemoglobinuria [PNH]$^{+}$) was available for the analysis. (A) all patients; (B), PNH^{+} patients; (C), PNH^{-} patients.

accurately determining the DRB1 alleles using high-resolution methods to clarify the role of HLA-DR15 in predicting a response to IST.

A higher frequency of HLA-DR15 among PNH^{+} patients in comparison to PNH^{-} patients has been reported by Maciejewsky et al. [26]. The present study confirmed this finding using a different flow cytometry assay that distinguished PNH^{+} patients from PNH^{-} patients using lower levels of glycosylphosphatidyl inositol-anchored protein-deficient cells than the assay used in the previous study. Our methods also identified a significant difference between DRB1*1501 and DRB1*1502 in the minimal expansion of PNH clones. The frequencies of both alleles increased in the PNH^{+} patients in comparison to normal controls, thus supporting the preliminary results of our study of 23 patients with refractory anemia [13]. However, only DRB1*1501 represented a genetic factor significantly associated with an increase in the proportion of PNH-type cells in AA patients in the present study because the frequency of DRB1*1502 was high in both PNH^{+} and PNH^{-} AA patients, thus indicating that the minimal expansion of PNH clones is not affected by DRB1*1502. Together with the difference in the response rate to IST between DRB1*1501^{+} and DRB1*1502^{+} AA patients, all these findings suggest that DRB1*1501 and DRB1*1502, therefore, play a different role in the pathogenesis of AA.

In AA patients carrying DRB1*1501, the presentation of autoantigen by this molecule may readily induce a cell-mediated attack against hematopoietic stem cells that may be associated with minimal expansion of a PNH clones. Previous studies have demonstrated that the presence of a CD4^{+} T-cell attack against hematopoietic stem cells allows the survival of PNH-type stem cells [27,28]. On the other hand, polymorphic gene alleles of myelosuppressive cytokines, in linkage disequilibrium with DRB1*1502 may predispose individuals with HLA-DRB1*1502 toward development of AA. In keeping with this hypothesis, a recent study on diabetes mellitus patients revealed that a haplotype of TNFa12-DRB1*1502 was, therefore, more frequent in patients likely to develop insulin-dependency than in those who do not develop insulin-dependency [29]. Several reports have demonstrated TNFa12 to be associated with a higher secretion of tumor necrosis factor-α [30].

HLA-DR15 molecules derived from DRB1*1502 differ from those derived from DRB1*1501 in only one amino acid at position 86 (valine for DRB1*1502 and glycine for DRB1*1501) of the β-chain [31]. This structural similarity indicates that antigenic epitopes presented by these molecules are common [32,33]. For most autoimmune diseases where DRB1*1501 is associated with susceptibility in patients from Western countries, DRB1*1502 is expected to play the same role as DRB1*1501 in Japanese patients. However, in Japanese patients with multiple sclerosis, the frequency of DRB1*1502 is not increased in comparison to that in the controls [34,35]. As a result, DRB1*1502 appears to contribute to development of some autoimmune diseases via different mechanisms to DRB1*1501. In AA patients carrying DRB1*1501, certain antigens of which presentation requires position 86 of the β-chain to be glycine may likely induce an immune system attack to hematopoietic progenitor cells. It is also possible that DRB5*0101 and DRB5*0102, which are in complete linkage disequilibrium with DRB1*1501 and DRB1*1502, respectively, in the Japanese population [19] may be responsible for the difference because DRB5*0101 differs from DRB5*0102 by three amino acids in the antigen-peptide binding domain.

Our data may be relevant to the management of AA. Although the incidence of HLA-DR15 is significantly higher in AA patients than in the normal controls, only DRB1*1501 was found to be a predictive marker for a good response to ATG plus CsA therapy. AA patients with DRB1*1502 who do not show an increased proportion of PNH-type cells may not benefit from IST. HLA-DR typing has been considered to be useful for predicting a good response to IST in AA patients [7,8], but this costly test may not be necessary in the circumstance where the highly sensitive flow cytometry is available because the presence of a small population of PNH-type cells is the only significant factor that affects the response to ATG plus CsA therapy based on the findings of our multivariate analysis. Prospective studies are called for to confirm these findings.

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ORIGINAL ARTICLE

Reduced-intensity unrelated cord blood transplantation for treatment of metastatic renal cell carcinoma: first evidence of cord-blood-versus-solid-tumor effect

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We report a 69-year-old man with cytokine-resistant metastatic renal cell carcinoma treated with reduced-intensity unrelated cord blood transplantation. The patient achieved durable donor engraftment with minimal graft-versus-host disease. The patient showed regression of metastatic disease, providing the first evidence of a graft-versus-tumor effect on a solid tumor resulting from cord blood graft.
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Keywords: reduced-intensity unrelated cord blood transplantation; metastatic renal cell carcinoma; graft-versus-tumor effect

Introduction

Metastatic renal cell carcinoma (RCC) is resistant to standard radiotherapy or chemotherapy, and patients with this disease have a poor outlook.[1] Although immunotherapy with cytokines such as interleukin 2 and interferon alpha can lead to regression of RCC in some patients, the response rate for these treatments remains around 10–20%, and response is usually temporary.[2] Recently, allogeneic stem cell transplantation utilizing mobilized peripheral blood from a matched donor has been investigated as an alternative immunotherapeutic strategy for the treatment of advanced RCC. The results of pilot reduced-intensity transplant trials for metastatic RCC are encouraging and show that responses can occur in patients with advanced metastatic disease that has failed to respond to conventional cytokine-based therapy.[1,3 15]

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Unrelated cord blood (UCB) is considered an alternative hematopoietic stem cell source for transplantation, and its use in adult patients with hematologic disorders is increasing.[16 21] Thus far, UCB transfer has not been attempted in patients with a solid-organ malignancy such as RCC. Here, we report a patient with metastatic RCC treated with reduced-intensity unrelated cord blood transplantation (RI-UCBT).

A 56-year-old man with clear cell RCC of his right kidney underwent a right nephrectomy in March 1991. Six years later, metastatic diseases were found in the right upper jaw and pancreas and were partially removed. The remaining metastases grew and new metastases developed in the left lung, left kidney, retroperitoneal space and subcutaneous space. The patient was treated with a 12-week course of combination therapy of subcutaneous interferon alpha 2 MU/m^2 and interferon gamma 2 MU/m^2 five times per week. However, these metastases showed a progressive increase in the size. Because of the low probability of response to further conventional treatment for metastatic RCC, the patient was referred to our institute in February 2004 at the age of 69 years. Then, serum LDH level was 286 IU/l (normal range, 0–250), hemoglobin level 10.6 g/dl, serum calcium level 9.3 mg/dl and erythrocyte sedimentation rate 38 mm/h. Reduced-intensity allogeneic stem cell transplantation was considered in order to decrease regimen-related toxicity, but because of the lack of a suitable donor candidate among his family members, unrelated RI-UCBT was planned. The patient gave written informed consent to participate in an institutional review board-approved investigational protocol designed to evaluate graft-versus-tumor (GVT) effects in metastatic RCC after nonmyeloablative allogeneic transplantation. The preparative regimen, which was based on a previous report,[22] consisted of cyclophosphamide, 50 mg/kg, on day −6, fludarabine, 40 mg/m^2, daily on days −6 to −2, and a single dose of 200 cGy of total body irradiation on day −1. UCB, phenotypically mismatched at one HLA-B antigen and one DRB1 antigen, was obtained through the Japanese Cord Blood Bank Network (J-CBBN). The patient received a UCB graft at a dose of 2.0×10^7 nucleated cells/kg of recipient body weight in

March 2004. To prevent rejection of the graft and graft-versus-host disease (GVHD), intravenous cyclosporine A (1.5 mg/kg b.i.d.) and oral mycophenolate mofetil (15 mg/kg b.i.d. until neutrophil engraftment) were started 3 days before transplantation. Granulocyte colony-stimulating factor (G-CSF) was initiated on day 1. The patient developed poor engraftment with at most 50% of peripheral blood granulocytes of donor origin. This resulted in graft rejection, with complete autologous recovery on day 41. One hundred and six days after first transplant, the patient received a second UCB graft from J-CBBN containing 2.2×10^7 nucleated cells/kg of recipient body weight, which was phenotypically mismatched at one HLA-B antigen and one DRB1 antigen. Conditioning therapy consisted of fludarabine, 25 mg/m^2, daily on days -7 to -3, melphalan, 80 mg/m^2, on day -2 and a single dose of 400 cGy of total body irradiation on day -1, as previously reported.[18] A continuous infusion of tacrolimus, 0.03 mg/kg, was started from 3 days before transplant for prophylaxis of GVHD and graft rejection. G-CSF was started on day 1. The patient tolerated the conditioning regimen well and exhibited rapid engraftment, with neutrophils rising above 5×10^8/l by day 15. Chimerism analysis of blood on day 20 after second transplant revealed 100% donor origin in both myeloid and T-lymphoid lineages. On day 47, grade II acute GVHD of the skin and gut developed. Acute GVHD improved rapidly after increasing doses of tacrolimus without corticosteroid therapy, but it became dependent on the treatment of tacrolimus. The tacrolimus was finally tapered off at 11 months, and thereafter no GVHD developed. Treatment response was evaluated monthly after transplantation according to the Response Evaluation Criteria in Solid Tumors (RECIST).[23] A computed tomography (CT) scan at 2 months showed substantial regression of metastasis in the left kidney and retroperitoneal space (Figure 1), and the patient was determined as partial remission (PR). The PR had lasted for 3 months until new metastatic lesions in the liver and pancreas appeared at 5 months after second transplantation, defined as progressive disease (PD). At the onset of PD, he had active GVHD of the gut, which was treated with oral tacrolimus alone. Metastatic lesions progressed in size very slowly until 18 months after second transplantation, but since then, they have been unchanged until the time of this writing. The association of the onset of GVHD with the development of PR as well as that of discontinuation of the immunosuppression with no further progression of disease is suggestive of a GVT effect in this patient. The patient continues to show a good performance 26 months after second transplantation without active GVHD.

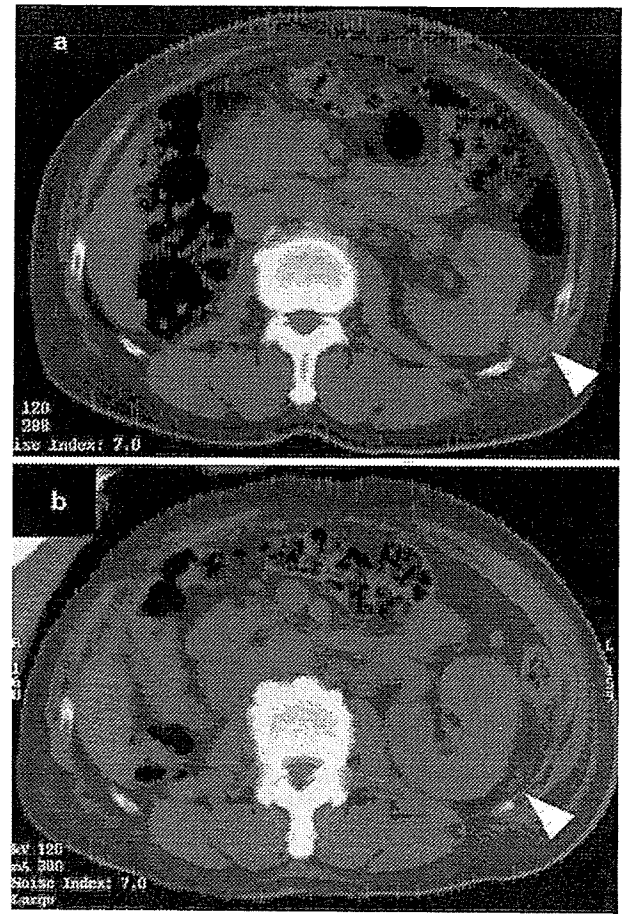

Figure 1 CT images of retroperitoneal metastasis (arrowheads) in a patient before second transplantation (a) and 2 months after second transplantation (b). Regression in the patient was concordant with the onset of acute GVHD of the skin and gut.

Discussion

Metastatic RCC is the solid tumor in which a GVT effect has been most expected. Childs *et al.*[3] and Childs and Otterud[24] have reported that of 50 patients with metastatic RCC who underwent allogeneic peripheral blood stem cell transplantation (PBSCT), 22 (44%) showed a disease response including four complete responses and 18 PRs, and five (10%) patients had a mixed response. However, the worldwide clinical experience of allogeneic SCT for metastatic RCC is limited, with approximately 200 cases reported in the literature.[3,15,24] One of the major restrictions of this approach is the requirement that transplant candidates have an HLA-identical related donor. This requirement may limit the use of allogeneic stem cell transplantation to a minority of patients with metastatic RCC. Our patient achieved long-term survival following RI-UCBT, despite the lack of a suitable donor candidate among his family members.

Cord blood, which is collected from the umbilical cord and placenta of healthy newborns, is an alternative source of hematopoietic stem cells.[25] Compared to adult peripheral blood or bone marrow, cord blood contains a greater proportion of highly proliferative hematopoietic progenitor cells,[26] which may account for myeloid and lymphoid reconstitution after cord blood transplantation (CBT) despite the presence of fewer cells (by 1–2 logs) in cord blood than in bone marrow or mobilized peripheral blood.

It was originally thought in CBT that the immunological naivety of cord blood lymphocytes[26] might produce a

lowered GVT effect at the expense of a lower GVHD incidence. However, clinical studies revealed similar rates of disease relapse and lower rates of acute and chronic GVHD in adult patients with hematologic malignancies receiving CBT compared to those receiving allogeneic bone marrow transplantation or PBSCT.[17,19,21] Although it remains unclear whether such favorable effects also occur in patients with metastatic RCC who undergo CBT, several observations support the hypothesis that similar alloimmune effects mediated by donor T cells could work in these patients.[24,27 30] Although the target antigens in GVT effects after allogeneic transplantation against metastatic RCC have not been determined, clinical and laboratory observations suggested that minor histocompatibility antigens (mHAs) could be mainly involved as target antigens in GVT effects for metastatic RCC after PBSCT, and donor T cells responding to mHAs could be generated.[24,27,28] The fact that cord blood can generate cytotoxic T cells specific for the mHA in the same way as peripheral blood and bone marrow[29,30] might imply that mHA-specific donor T cells contributive to a GVT effect against metastatic RCC are inducible in a patient receiving a cord blood graft as well.

As allogeneic stem cell transplantation is associated with many and sometimes severe toxic effects, we used a reduced-intensity conditioning regimen as described in previous reports,[18,22] which included low-dose total body irradiation in combination with cyclophosphamide and fludarabine or with melphalan and fludarabine. This RI-UCBT regimen proved to be well tolerated and achieved durable donor engraftment with minimal GVHD. Although our patient required a second RI-UCBT because of graft refection after first RI-UCBT, the demonstrated feasibility of secondary transplantation may be of benefit in the treatment of older cancer patients with RI-UCBT. Of note, the observation in the patient that retroperitoneal and renal metastasis regressed, despite a mixed response, provides the first evidence of a GVT effect by a cord blood graft on RCC.

The advantages of CBT are the immediate availability of cells, the absence of risk to the donor and a lower need for HLA compatibility between the donor and the recipient.[16 22] Because of the establishment of many cord blood banks, nearly every patient can find a potential cord blood graft, suggesting that CBT could substantially expand the use of allogeneic transplantation in patients with metastatic RCC. Despite these potential advantages, there are several disadvantages such as susceptibility to graft rejection, prolonged recovery of hematopoiesis and unavailability of donor lymphocyte infusions. A clinical study focusing on minimizing toxicities and controlling infectious complications as well as enhancing GVT effects is needed to optimize the success of CBT for treatment of advanced RCC.

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Progress in Hematology

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Clinical Significance of a Small Population of Paroxysmal Nocturnal Hemoglobinuria–Type Cells in the Management of Bone Marrow Failure

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Abstract

Although increased blood cell deficiency of glycosyl phosphatidylinositol–anchored membrane proteins has often been detected in patients with aplastic anemia (AA) and myelodysplastic syndrome (MDS), the clinical significance of such paroxysmal nocturnal hemoglobinuria (PNH)-type cells remains to be elucidated. We established a sensitive flow cytometric assay capable of detecting less than 0.01% of $CD59^-CD55^-$ blood cells in a sample and used the assay to examine a large number of patients with bone marrow failure. An increase in the proportion of PNH-type cells was detectable in approximately 60% of all AA patients and in 20% of all refractory anemia (RA)-MDS patients. The increase was undetectable in patients with RA with an excessive number of blasts, acute myelogenous leukemia, multiple myeloma, or systemic lupus erythematosus. Our study showed that the presence of an increased number of PNH-type cells was predictive of a good response to immunosuppressive therapy and a favorable prognosis among patients with recently diagnosed AA and RA. A sensitive flow cytometric analysis for detection of a small population of PNH-type cells in peripheral blood cells is one of the most important examinations in the management of bone marrow failure.
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Key words: Paroxysmal nocturnal hemoglobinuria–type cells; Bone marrow failure

1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia characterized by an increased number of glycosyl phosphatidylinositol (GPI)-anchored membrane protein–deficient cells derived from hematopoietic progenitor cells with a *PIG-A* gene mutation [1]. A small population of GPI-deficient blood cells are often detected in patients with bone marrow failure who do not have clinical or laboratory signs of hemolysis [2,3]. This type of marrow failure has been defined as subclinical PNH (PNH-sc) by the International PNH Interest Group [4]. In several studies investigators have attempted to clarify the clinical significance of such PNH-type cells in bone marrow failure patients. Some studies have revealed that the presence of PNH-type cells in patients with myelodysplastic syndrome (MDS) [5] and aplastic anemia (AA) [6] is predictive of the response to immunosuppressive therapy (IST). Other studies, however, have not confirmed the predictive value of PNH-type cells [7,8]. This discrepancy may be due to differences in the sensitivity and specificity of the flow cytometric assays used in previous studies to detect PNH-type cells.

We have been using a high-sensitivity flow cytometric assay to detect PNH-type cells in the peripheral blood of patients with various types of bone marrow failure and thus postulated the significance of an increase in the proportion of PNH-type cells as a marker of the immune pathophysiology of AA and refractory anemia (RA)-MDS [9-11]. In this review, we summarize our experience over the past 6 years in the detection of PNH-type cells in patients with various hematologic diseases. We emphasize the importance of using a high-sensitivity flow cytometric assay to detect small populations of PNH-type cells in the management of bone marrow failure.

2. Detection of a Small Population of PNH-Type Cells

A small number of $CD55^-CD59^-$ cells can be detected in healthy individuals [12,13]. When we used a 2-color flow cytometric assay to examine 1,000,000 peripheral blood granulocytes from 5 healthy individuals, 12 to 28 $CD55^-CD59^-$ cells

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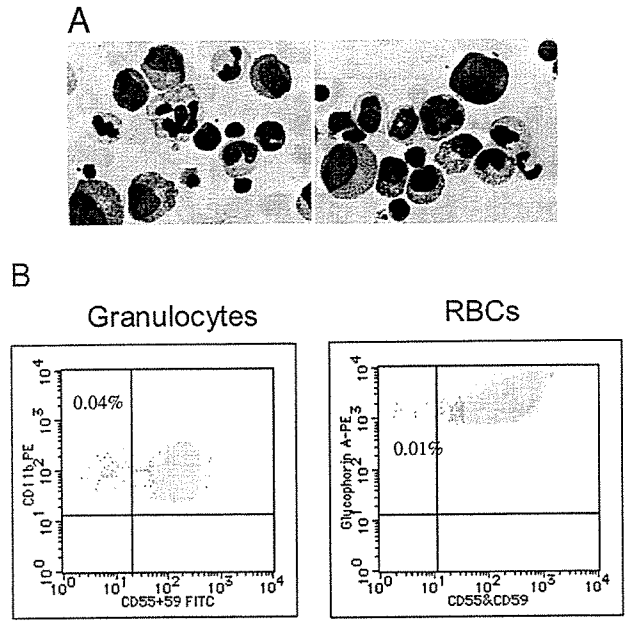

Figure 1. Bone marrow morphology and flow cytometric examination of a typical patient with subclinical paroxysmal nocturnal hemoglobinuria. Bone marrow photomicrograph (A) and results of high-sensitivity flow cytometric analysis (B) of a 28-year-old woman whose condition was diagnosed as myelodysplastic syndrome refractory cytopenia with multilineage dysplasia. RBCs indicates red blood cells; FITC, fluorescein isothiocyanate.

were detected [9]. When we examined 100,000 granulocytes, an appropriate number for routine examination of patient samples, from 68 healthy individuals, 26.5% of the cells exhibited either 1 or 2 $CD55^-CD59^-$ granulocytes, and 50.0% exhibited 1 or 2 $CD55^-CD59^-$ red blood cells (RBCs) [10]. No healthy individual had 3 or more $CD55^-CD59^-$ cells per 100,000 granulocytes and 100,000 RBCs. On the basis of these results, we defined the presence of more than 0.003% $CD55^-CD59^-$ cells among both granulocytes and RBCs as a definite increase in the number of PNH-type cells.

2.1. Importance of Detecting a Minor Population of PNH-Type Cells

The best example for demonstrating the significance of PNH-type cells is moderate AA mimicking RA-MDS. A slight increase in the proportion of PNH-type cells is often detected in patients with this type of bone marrow failure [10]. Because of the presence of moderate signs of dysplasia and normocellularity in iliac bone marrow, the condition of these PNH-sc patients usually is diagnosed as either RA or refractory cytopenia with multilineage dysplasia (RCMD) rather than moderate AA.

Figure 1A shows the marrow characteristics of a typical PNH-sc patient. Because of the presence of erythroid and myeloid dysplasia and of normocellularity in the bone marrow, the condition of this 28-year-old woman was diagnosed as RCMD during pregnancy. The physician in charge planned to treat the patient with allogeneic transplantation from an unrelated donor after delivery of the infant. However, our flow cytometric analysis revealed a small population of $CD55^-CD59^-$ cells in both the granulocytes and the RBCs (Figure 1B). We therefore treated the patient with antithymocyte globulin plus cyclosporine after delivery. Her blood cell counts completely normalized within 1 year after IST. The patient was in complete remission more than 5 years after treatment. Recent bone marrow aspiration revealed no signs of dysplasia.

This patient was about to be treated improperly. PNH-sc patients are likely to be treated with cytotoxic drugs or stem cell transplantation from unrelated donors or to be observed with supportive care unless a high-sensitivity flow cytometric assay is used to examine their blood for the presence of a small population of PNH-type cells.

2.2. Reasons for High Sensitivity of the Flow Cytometric Assay

Conventional flow cytometric assays have defined the presence of 1% or more $CD55^-CD59^-$ cells as indicating a significant increase in the proportion of PNH-type cells, because $CD55^-CD59^-$ cells up to 1% of the total granulocyte and RBC counts are frequently detected in healthy individuals [14]. We used a highly sensitive flow cytometric assay to examine the peripheral blood of more than 600 patients with bone marrow failure for the presence of PNH-type cells. As shown in Figure 2, 56.4% of AA and 19.4% of RA patients were judged to have PNH-sc. Notably, more than 80% of the PNH-sc patients had less than 1% PNH-type cells. Therefore these patients, who are potentially responsive to IST, are overlooked on the basis of findings of conventional flow cytometry.

2.3. Accurate Detection of Small Populations of PNH-Type Cells

In flow cytometry, damaged cells and dead cells can be often be mistakenly identified as $CD55^-CD59^-$ cells owing to

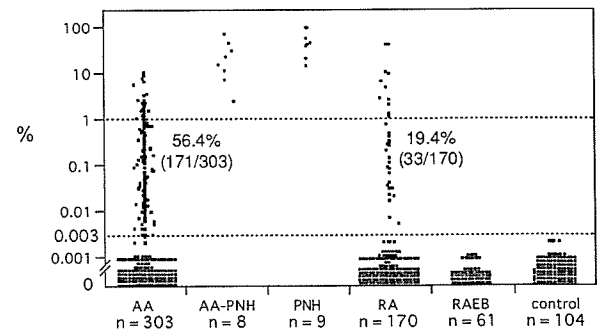

Figure 2. Proportions of PNH-type cells in $CD11b^+$ granulocytes of various patients. Squares indicate proportion of $CD55^-CD59^-CD11b^+$ granulocytes determined with high-sensitivity flow cytometric analysis; AA, aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; RA, refractory anemia; RAEB, RA with excess of blasts.

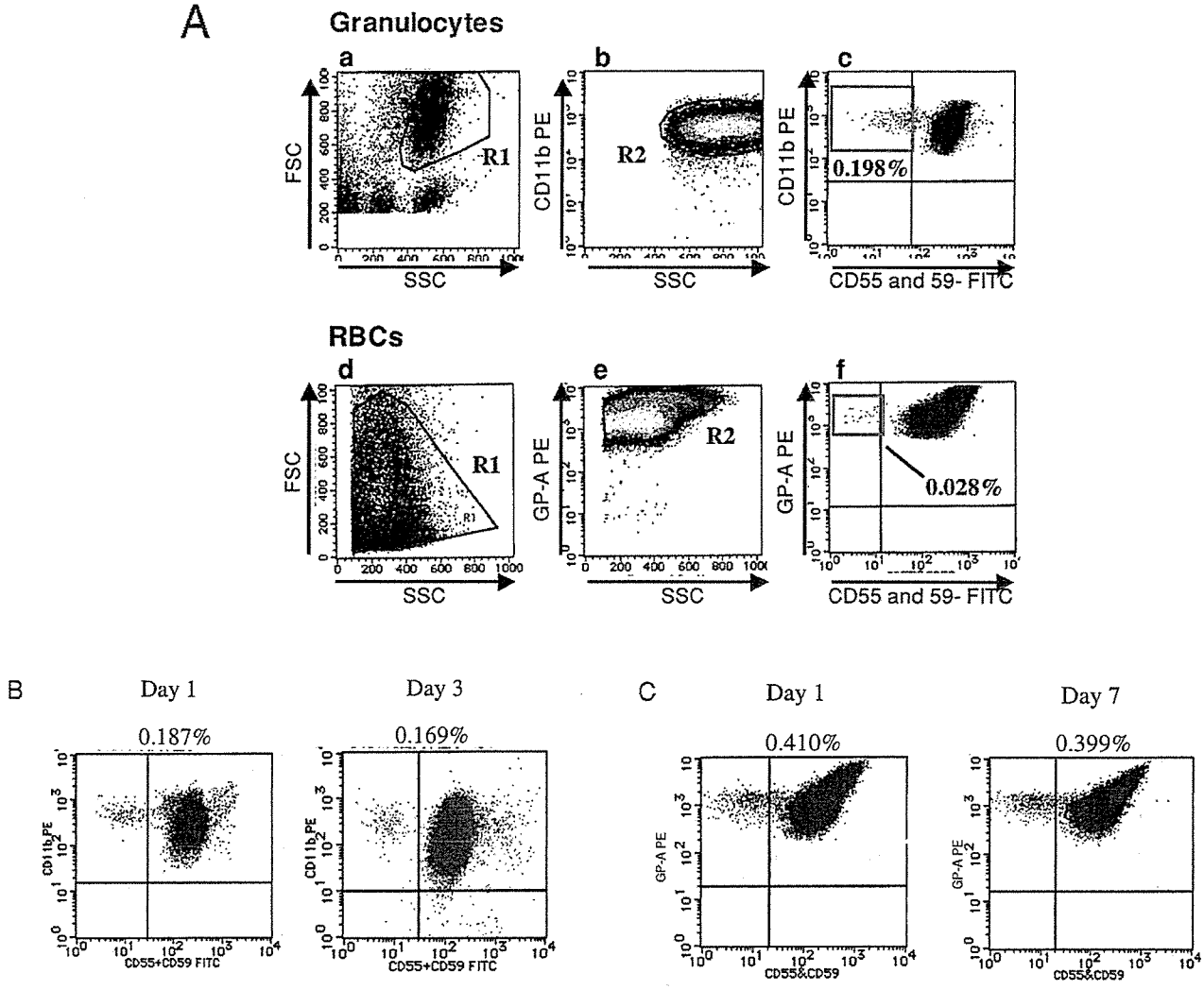

Figure 3. High-sensitivity flow cytometric analysis. A, Example of analysis on granulocytes (A, B, C) and red blood cells (RBCs) (D, E, F) of a patient with aplastic anemia and an increased proportion of paroxysmal nocturnal hemoglobinuria–type cells. Gates were set up to exclude SSC^{dim} (A) and $CD11b^{dim}$ (B) granulocytes and glycophorin A^{dim} (E) RBCs. FSC indicates forward scatter; SSC, side scatter; GP-A PE, glycoprotein A phycoerythrin; FITC, fluorescein isothiocyanate. B, Influence of time from blood collection to treatment of granulocytes with monoclonal antibodies. C, Influence of time from blood collection to treatment of RBCs with monoclonal antibodies.

poor binding of antibodies to these GPI-anchored proteins. It is necessary to eliminate the damaged and dead cells from analysis to reduce the cutoff value to less than 0.01%. By careful gating, as shown in Figure 3A, our sensitive flow cytometric analysis excludes SSC^{dim} and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs on the histograms [15].

The time from blood collection to treatment of blood cells with monoclonal antibodies is an important factor influencing the results of flow cytometry. Granulocytes have to be treated within 24 hours after blood collection, because CD11b expression by granulocytes diminishes over time (Figure 3B). On the other hand, the time period from 7 to 14 days after blood collection does not affect the results of RBC analysis (Figure 3C). Only 10% of patients have an increase in the proportion of PNH-type cells in granulocytes but not in RBCs [15]. Accordingly, 90% of cases of PNH-sc can be precisely diagnosed with examination of RBCs alone.

3. Clinical Significance of PNH-Type Cells in Patients with Recently Diagnosed AA

We recently compared the IST responses of 83 AA patients who had an increased proportion of PNH-type cells (PNH^+ patients) with that in 39 AA patients who did not have such an increase (PNH^- patients) [15]. Figure 4A shows the cumulative incidence of achieving a partial response to IST as defined by Camitta [16]. PNH^+ patients had a significantly better and faster response than PNH^- patients. Although the

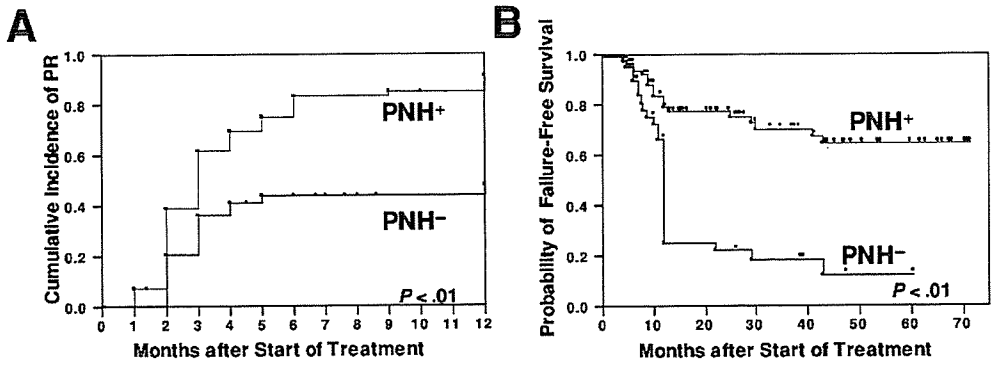

Figure 4. Response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Cumulative incidence of overall responses (A) and failure-free survival (B) in patients with an increased proportion of paroxysmal nocturnal hemoglobinuria–type cells (PNH^+) and patients without such an increase (PNH^-). PR indicates partial response.

overall 5-year survival rate was similar for PNH^+ (77.0%) and PNH^- (70.8%) patients, the probability of surviving failure free for 5 years was markedly higher among PNH^+ patients (64.2%) than PNH^- patients (12.3%) (Figure 4B). These results indicate that a minor population of PNH-type cells is a reliable marker of a positive IST response and a favorable prognosis among patients with AA. The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in AA thus needs to be evaluated with a prospective study. A cooperative study led by the Intractable Disease Study Group of Japan and other referring institutions is underway in Japan.

4. Increase in Proportion of PNH-Type Cells in Diseases Other than AA and RA

Studies have revealed an increased number of PNH-type cells in patients with systemic lupus erythematosus (SLE) [17] and multiple myeloma (MM) [18]. Using high-sensitivity flow cytometry, we analyzed the cases of 43 SLE patients and 14 MM patients and found no small populations of PNH-type cells in any of these patients. As a result, an increase in the proportion of PNH-type cells is thus considered a characteristic of bone marrow failure.

Among bone marrow failure patients, an increased number of PNH-type cells was undetectable in any of 61 patients with RA with excess of blasts or 11 patients with acute myelogenous leukemia (Figure 2). These findings are in line with our observation that clonal hematopoiesis arose more frequently in PNH^- than in PNH^+ AA or RA patients [11]. Accordingly, the presence of PNH-type cells in bone marrow failure thus represents a benign feature of bone marrow failure.

5. Bone Marrow Cellularity in PNH-sc

One of the main reasons for the misdiagnosis of PNH-sc as MDS is that bone marrow aspirate and biopsy specimens from PNH-sc patients often show either normocellularity or hypercellularity. Although most areas of bone marrow are replaced with fat tissue, PNH-sc patients usually have residual hematopoietic nests that produce confusing results during pathological diagnosis. Figure 5A shows a sagittal magnetic resonance image (MRI) of the pelvis from a PNH-sc patient. In this T1-weighted image, dark (cellular) spots are present on the white (fatty) background. Biopsy specimens acquired by chance from the cellular spot are judged normocelluar. A T1-weighted MRI of the thoracic and lumbar vertebrae of the same patient shows fatty marrow with a few cellular spots (Figure 5B), indicating marrow cellularity to be low on the whole. As a result, the bone marrow cellularity of marrow failure patients cannot be determined with bone marrow biopsy alone. MRIs of the thoracic and lumbar vertebrae also must be obtained, particularly when the bone marrow biopsy findings from patients with pancytopenia show either normocellularity or hypercellularity. Inhomogeneous MRI patterns are commonly seen not only in MDS patients [19] but also in PNH-sc patients. A similar MRI pattern of a PNH-sc patient has been reported by Kouba et al [20].

6. Conclusions

Detection of a small population of PNH-type cells is the most fundamental examination for assessing the pathophys-

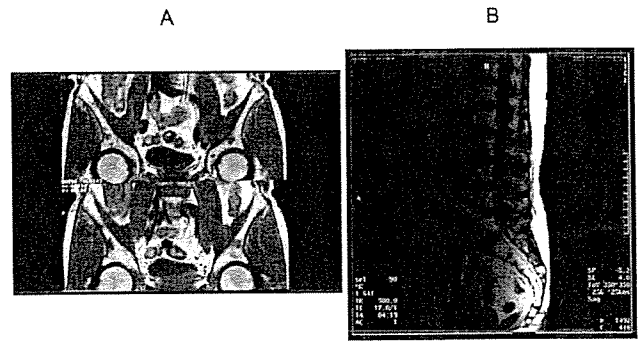

Figure 5. Magnetic resonance images of a patient with subclinical paroxysmal nocturnal hemoglobinuria. A, T1-weighted image of the pelvis. B, T1-weighted image of the thoracic and lumbar vertebrae.

iology of bone marrow failure. High-sensitivity flow cytometry is easily performed. A PDF file that describes use of flow cytometry detection of small populations of PNH-type cells in the peripheral blood can be downloaded from http://web.kanazawa-u.ac.jp/~med18/.

The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in bone marrow failure warrants a worldwide prospective study with non-Japanese patients with AA.

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TRANSLATIONAL AND CLINICAL RESEARCH

HOX Decoy Peptide Enhances the Ex Vivo Expansion of Human Umbilical Cord Blood CD34$^+$ Hematopoietic Stem Cells/Hematopoietic Progenitor Cells

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Key Words. Ex vivo expansion • Hematopoietic stem/progenitor cells • HOX • Peptide mimetics

ABSTRACT

HOX transcription factors play important roles in the self-renewal of hematopoietic cells. HOX proteins interact with the non-HOX homeobox protein PBX1 to regulate, both positively and negatively, the expression of target genes. In this study, we synthesized a decoy peptide containing the YPWM motif from HOX proteins (decoy HOX [decHOX]), which was predicted to act as a HOX mimetic, and analyzed its effects on self-renewal of human cord blood CD34$^+$ cells. We were able to deliver decHOX into approximately 70% of CD34$^+$ cells. By examining the expression of HOX target genes c-*myc* and *p21$^{waf1/cip1}$*, we confirmed that decHOX enhanced HOX functions. After 7 days of culture in serum-free medium containing a cytokine cocktail, cultures treated with decHOX had approximately twofold-increased numbers of CD34$^+$ cells and primitive multipotent progenitor cells compared with control cells. Furthermore, decHOX-treated cells reconstituted hematopoiesis in nonobese diabetic/severe combined immunodeficiency mice more rapidly and more effectively (more than twofold greater efficiency, as determined by a limiting dilution method) than control cells. decHOX-treated cells were also able to repopulate secondary recipients. Together, these results indicate that in combination with growth factors and/or other approaches, decHOX might be a useful new tool for the ex vivo expansion of hematopoietic stem/progenitor cells. STEM CELLS *2006;24:2592–2602*

INTRODUCTION

Human umbilical cord blood (CB) is a useful source of hematopoietic stem cells (HSCs) for transplantation. In fact, during the last few years, an increasing number of patients have received CB transplants [1]. However, clinical applications of CB are inevitably limited by the fact that the number of HSCs in each CB sample is insufficient for many adult patients. Also, compared with transplantation of HSCs from the bone marrow or HSCs mobilized into peripheral blood, the recovery of hematopoiesis is rather delayed in patients receiving CB transplants, partly because of the insufficient number of transplanted HSCs and progenitor cells and the persistent quiescence of CB HSCs that sometimes accompanies lethal complications [1]. Therefore, it is of particular interest to expand CB HSCs ex vivo and to develop strategies for hastening hematopoietic recovery after CB transplantation in vivo [2]. Regarding strategies for ex vivo expansion, the most important problem is to preserve the functions and properties of HSCs, that is, self-renewal and multipotency, during culturing. At present, the use of cytokines is the most promising and practical strategy for this purpose. To establish the culture conditions most suitable for expansion of HSCs, a number of investigators have used various cytokine combinations [2, 3]. When their effects were compared by long-term reconstitution assays in transplanted mice, the combination of stem cell factor (SCF), FLT3 ligand (FL), thrombopoietin (TPO), and IL-6/soluble IL-6 receptor (sIL-6R) was found to expand HSCs most efficiently, with a 4.2-fold increase in severe combined immunodeficient (SCID)-repopulating cells (SRC) [4]. Several patients have received the transplantation with cytokine-expanded CB HSCs, and these cells were transplanted without serious toxicities [5, 6]. However, although increased numbers of infused CB HSCs were shown to correlate with good outcomes, cytokine-expanded CB HSCs did not shorten the nadir period after transplantation, indicating the

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limited usefulness of cytokines for ex vivo expansion of CB HSCs. Thus, further improvement in ex vivo expansion procedures is necessary to prepare more efficient HSCs.

During the last few years, several molecules that can contribute to HSC self-renewal have been identified and characterized. These include external signaling molecules such as Wnt [7–10], bone morphogenic protein (BMP) [11], Sonic hedgehog (SHH) [12], and Notch ligands [13–15]. Furthermore, endogenous transcriptional modulators such as HOXB4 and Bmi-1 have been shown to be important for HSC self-renewal [16–18]. Among these, HOXB4 is of particular interest because it promotes prominent expansion of HSCs without causing leukemia. When HOXB4 was introduced into murine or human HSCs by gene transfer or protein delivery, these HSCs could be expanded without losing their normal potentials for differentiation into all lineages and for long-term repopulation, with a few exceptions [16, 19–22]. In addition to HOXB4, other HOX homeobox transcription factors play important roles in the proliferation and differentiation of hematopoietic cells [23, 24]. For example, HOXA9 regulates HSCs by mediating the expression of a variety of gene families [25, 26]. HOXA5/A10 and HOXB6 induce differentiation toward the myelomonocytic or erythroid lineage, respectively [27–30]. Furthermore, other HOX transcription factors, especially paralogous groups from A, B, and C, are expressed in normal hematopoietic cells; however, their physiological functions have not been elucidated.

HOX proteins have been demonstrated to interact with non-HOX homeobox family proteins (i.e., PBX and MEIS) at the DNA sequence 5′-TGATNNAT(G/A)(G/T)-3′ in the regulatory region of target genes [31, 32]. These protein complexes regulate target gene expression both positively and negatively, dependent on binding to coactivators or corepressors such as CBP/p300, histone deacetylases, or NcoR/SMRT [33–36]. For a subset of HOX proteins, the formation of a HOX-PBX-DNA ternary complex is mediated through both the HOX homeodomain and a short, conserved YPWM motif located just upstream of the HOX homeodomain [37, 38]. The interaction between the YPWM motif of HOX and the third α-helix in the homeodomain of PBX1 is thought to modify HOX-PBX1 DNA-binding affinity and transcriptional activity [39–41]. In addition, it was reported that PBX1 expressed in HSCs is a negative regulator of HOXB4-mediated self-renewal of HSCs [42]. Consistent with this report, a very recent study demonstrated that although DNA-binding activities are necessary for HOXB4 to expand HSCs ex vivo, the interaction with PBX1 is dispensable for this function [43].

In an attempt to expand potent CB HSCs with high efficiency, we synthesized a peptide containing the YPWM motif from HOX, which was predicted to modify HOX function by inhibiting binding between the YPWM motif in endogenous HOX and the PBX1 homeodomain. Here we show that this decoy HOX (decHOX) peptide augments the cytokine-dependent ex vivo expansion of CD34-positive hematopoietic stem/progenitor cells ($CD34^+$ hHSCs/HPCs), and these cells have the ability to reconstitute hematopoiesis more effectively and rapidly in mice that received transplants.

Materials and Methods

Peptide Synthesis

Peptides were synthesized at Greiner Bio-One (Tokyo, Japan, http://www.gbo.com/en) with purities of more than 95%. Synthetic peptides were lyophilized and stored at −20°C until use.

Reagents and Antibodies

Recombinant human SCF, TPO, IL-6, and sIL-6R were provided by Kirin Brewery (Tokyo, Japan, http://www.kirin.co.jp/english/). Recombinant human FL was purchased from R&D Systems Inc. (Minneapolis, http://www.rndsystems.com). Anti-asialo-GM1 antibody (Ab) was purchased from Wako Chemical (Osaka, Japan, http://www.wako-chem.co.jp/english). Antibodies (Abs) against HOXB4 (N-18) and PBX1 (P-20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, http://www.scbt.com).

Plasmids

The expression vectors for HOXB4 and PBX1 were kindly provided by Dr. R. K. Humphries (British Columbia Cancer Agency, Vancouver, BC, Canada) and Dr. M. Featherstone (McGill University, Montreal, QC, Canada), respectively.

Preparation of Glutathione *S*-Transferase Fusion Proteins

Mutants of PBX1 were generated by polymerase chain reaction (PCR) and subcloned into pGEX-5X-1 (GE Healthcare Bio-science Corp., Piscataway, NJ, http://www.gehealthcare.com). Glutathione *S*-transferase (GST)-PBX1 fusion proteins were produced in *Escherichia coli* and purified as described previously [44].

In Vitro Binding Assays Using the BIAcore System

To assess in vitro binding between decHOX and PBX1, we used the BIAcore system (Biacore AB, Uppsala, Sweden, http://www.biacore.com/lifesciences/index.html). The details of this system are described elsewhere [45]. Briefly, we immobilized decHOX on the surface of CM5 sensor chips. Solution containing each GST-PBX1 fusion protein was injected over the sensor chips. Binding kinetics were monitored by changes in the weight of sensor chips and evaluated as arbitrary resonance units (RUs).

Mice

Nonobese diabetic/Shi-severe combined immunodeficient (NOD/SCID) mice, which lack mature lymphocytes and circulating complement proteins and have defective macrophages, were obtained from Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). The mice were kept in microisolator cages on laminar flow racks in a clean experiment room and fed an irradiated, sterile diet and autoclaved, acidified water. Animal care was in accordance with institutional guidelines.

Cell Preparation

Human umbilical CB was obtained from normal, full-term deliveries upon obtaining informed consent. After sedimentation of the red blood cells with 6% hydroxyethyl starch (HES), mononuclear cells (MNCs) were separated by Ficoll-Hypaque density gradient centrifugation. $CD34^+$ cells were purified from MNCs using a MACS Direct CD34 Progenitor Cell Isolation Kit