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We clearly showed the presence of GPC3 peptide–specific CTLs in peripheral blood, and showed that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination. The evidence in this study serves as a proof-of-concept for immunotherapy using tumor antigen–specific CTLs. However, we did not confirm that the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide–specific CTLs. We are currently initiating a pilot study of liver biopsies carried out before and after GPC3 peptide vaccination for advanced HCC to determine whether tumor-infiltrating lymphocytes are indeed GPC3 peptide–specific CTLs.

No complete responses were observed when GPC3 peptide vaccination was used as the sole therapy for advanced HCC. To-date, there has been no report of an adequate antitumor efficacy of immunotherapy in clinical trials involving patients with advanced HCC; however, immunotherapy, as an adjuvant after surgical resection, is expected (38). On the basis of this study, we have begun a phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC and have also planned combinatorial approaches with chemotherapy.

In conclusion, this phase I clinical trial of a GPC3-derived peptide vaccine showed the vaccination to be safe and indicated a plethora of immunologic responses. This study also showed that GPC3-specific CTL frequency was correlated with OS in patients with advanced HCC who received the GPC3 peptide vaccine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Kuronuma, T. Takayama, K. Uesaka, J. Furuse, T. Nakatsura
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sawada, T. Yoshikawa, D. Nobuoka, H. Shirakawa, Y. Motomura, H. Ishii, K. Nakachi, M. Konishi, S. Takahashi, N. Gotohda, J. Furuse, T. Kinoshita, T. Nakatsura
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Writing, review, and/or revision of the manuscript: Y. Sawada, T. Yoshikawa, D. Nobuoka, T. Takayama, J. Furuse, T. Nakatsura
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Furuse
Study supervision: T. Nakagohri, T. Takayama, K. Yamao, J. Furuse
Monitoring and evaluation of data and safety of the study: K. Uesaka

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Novel and rapid enumeration method of peripheral blood stem cells using automated hematology analyzer

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PBSCT, HPC, automated hematology analyzer, hematopoietic stem cell transplantation

SUMMARY

Introduction: The number of infused $CD34^+$ cells is crucial to the success of peripheral blood stem cell transplantation (PBSCT). Here, we present, for the first time, a new method of enumerating hematopoietic progenitor cells (HPCs) for PBSCT.

Method: This novel method is based on hemolysis and chemical staining, followed by flow cytometry-based optical detection, conducted using an automated hematology analyzer (XN series, Sysmex). $CD34^+$ cells and HPCs were compared in 76 granulocyte colony-stimulating factor (G-CSF)-mobilized blood or apheresis samples taken from healthy donors (n = 18) or patients undergoing autologous PBSCT (n = 6).

Results: There was a strong correlation between the numbers of HPCs and $CD34^+$ cells (R^2 = 0.958). The expected total number of HPCs in the final products, which was estimated from HPCs in pre-apheresis PB or mid-apheresis products, also correlated well with the total number of $CD34^+$ cells in the final products. The change in HPCs in PB closely resembled that of $CD34^+$ cells during mobilization. Experiments using immunomagnetic beads suggested that the majority of $CD34^+$ cells existed in HPCs, and vice versa.

Conclusion: Hematopoietic progenitor cells may serve as surrogates for $CD34^+$ cells in PBSCT. However, further investigations are required to verify this.

INTRODUCTION

The number of infused CD34$^+$ cells is crucial to the success of peripheral blood stem cell transplantation (PBSCT) [1–3]. Although the ability to count CD34$^+$ cells currently depends solely on flow cytometric analysis, the complexity of the procedure and cost of reagents, including monoclonal antibodies, are major disadvantages. Biochemical examination using the hemolysis reaction and staining with a specific dye is another tool that has been used for many years as a mainstay for cell classification; most automated hematology analyzers utilize specific biochemical properties for counting and classifying blood cells [4, 5]. As blood cells mature and differentiate, the constituents of the cell membrane, cytoplasm, and intracellular granules change, and hemolysis-resistant properties depend on the degree of maturation. This is the basic idea behind the identification of immature cells with an automated hematology analyzer without using monoclonal antibodies [6].

Recently, this technology has been applied in practical use as the hematopoietic progenitor cell (HPC) counting method on the Sysmex SE-9000 (SE) and XE-2100 (XE) automated hematology analyzers. The SE/XE analyzers quickly estimate the number of immature cells, referred to as HPCs, at a very low cost [6, 7]. The number of SE/XE-determined HPCs in the peripheral blood (PB) is used to determine the optimal timing of PBSC collection [8–22]. The SE/XE-determined number of HPCs has also been shown to correlate well with the CD34$^+$ cell yield in apheresis products [8, 9, 13, 16–18]. However, the number of SE/XE-determined HPCs is limited as a substitute for the CD34$^+$ cell count [20, 21] because it is likely to be affected by coexisting immature cells (e.g., immature granulocytes), resulting in under- or overestimation of the number of HPCs in some situations [12–14, 22].

The newly launched XN-series model automated hematology analyzer (Sysmex Corporation, Kobe, Japan) is equipped with a white precursor cell (WPC) channel to detect immature cells, such as myeloblasts and abnormal lymphocytes. The measuring principle of this channel utilizes the optical detection system and general flow cytometry. In this channel, two reagents are used, that is, the lysis reagent (Lysercell WPC, Sysmex) containing a polyoxyethylene nonionic surfactant and the stain reagent (Fluorocell WPC, Sysmex) containing a polymethine dye. We optimized the hemolysis conditions, including reaction time, temperature, to identify PBSCs with reference to CD34$^+$ cells. Finally, we developed a new technology for counting HPCs based on hemolysis reactions with the surfactant and chemical staining with a specific dye.

The purpose of this study was to evaluate the XN-determined HPCs in reference to CD34$^+$ cells. This is the first report to investigate this topic.

MATERIALS AND METHODS

Study design

Healthy PBSCT donors and patients with hematological malignancies undergoing autologous PBSCT at the National Cancer Center Hospital, Japan, were enrolled into this study. PBSC and PB samples were taken from each donor or patient during granulocyte colony-stimulating factor (G-CSF) mobilization or the bone marrow recovery phase after chemotherapy. HPCs and CD34$^+$ cells were examined in the same fresh samples and were subsequently compared. The primary objective was to examine whether there was a correlation between HPCs and CD34$^+$ cells in PB and PBSC samples. We also investigated whether it was possible to predict the total number of CD34$^+$ cells in the final product from HPCs in the PB immediately prior to the start of apheresis and from HPCs in the PBSC sample taken during an apheresis session. The kinetics of HPCs and CD34$^+$ cells were also examined by taking PB samples at several time points during PBSC mobilization to examine whether the correlation between these two markers was constant at all time points. The relationship between the cell populations defined by HPCs and CD34$^+$ cells was investigated using CD34$^+$ cell-enriched or cell-depleted PBSC samples, which were obtained by isolation with immunomagnetic beads. This study was approved by the institutional review board, and informed consent was obtained from each patient or donor.

PBSC mobilization and apheresis

Healthy donors were administered G-CSF subcutaneously at a dose of 10 μg/kg per day, and apheresis was started on the fourth or fifth day until the target total

number of 2 × 10^6 $CD34^+$ cells per kg of patient body weight was obtained. Patients underwent apheresis until the same target number of $CD34^+$ cells was obtained during the bone marrow recovery phase after chemotherapy under the support of intravenous G-CSF (5 μg/kg/day). Apheresis was performed using the COBE Spectra cell separator (Terumo BCT, Lakewood, CO, USA) with 10 L of blood processed in each subject. An acid–citrate–dextrose A (ACD-A) solution (Terumo, Japan) mixed with heparin sodium at a concentration of 5 U/mL was used for anticoagulation during apheresis.

Measurements of HPCs

Hematopoietic progenitor cells were measured using the Prototype XN-series automated hematology analyzer (Sysmex) with the WPC channel. Additional software for HPC counting was installed on the instrument without modifying the hardware or reagents of the released product model. The lysis reagent for this channel contained a surfactant. Lipid components, constitutive elements of the cell membrane, were extracted by the surfactant, and minute pores were formed, resulting in reduced stability of the cellular structure and shrunk or broken cells [23–25]. Then, dyes migrated into the cells through the openings formed by the surfactant and bound to nucleic acids or cytoplasmic organelles. The greater the damage to the leukocytes, the more easily they were stained. The lipid content of the membranes of white blood cells (WBCs) has been shown to increase with maturation, and mature neutrophils contain the highest levels of phospholipids [26–28]. Therefore, mature neutrophils were more likely to be damaged than immature leukocytes by incubation with reagents containing a surfactant. The rising fluorescence intensity of cells after incubation was associated with the degree of cell membrane damage. Thus, these biochemical reactions highlighted cellular characteristics associated with the membrane, cytoplasm, and intracellular granules of each cell type on the basis of their unique staining patterns and morphological features. Finally, three signals were produced by the optical detection system: forward scattered light, providing information on cell size; side scattered light, providing information on internal cell structure; and side fluorescence, providing information on the degree of dye staining. The results were shown as the HPC concentration (cells/μL) on the screen with a scattergram of the HPC analysis. HPCs were defined as dots that showed higher light intensity than lymphocytes in the forward scatter, which reflected the cell size, and equal light intensity as lymphocytes in the side scatter, which reflected the complexity caused by the presence of intracellular granules. In addition, they were also defined as dots that showed lower light intensity than lymphocytes in the side fluorescence scatter, which reflected the degrees of resistance to the surfactant and maturation (Figure 1). In the WPC channel, 4 μL of sample was used for HPC analysis, and the whole assay required <200 μL volume, <5 min time, and no monoclonal antibodies.

Measurements of $CD34^+$ cells

$CD34^+$ cells were quantitated using the dual platform method according to the protocol of the International Society of Hematotherapy and Graft Engineering (ISHAGE) [29, 30]. Briefly, cells were double-labeled with phycoerythrin (PE)-conjugated anti-CD34 and fluorescein isothiocyanate (FITC)-conjugated anti-CD45 monoclonal antibodies (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson). The number of $CD34^+$ cells was calculated from the percentage of $CD34^+$ cells with reference to $CD45^+$ and WBC counts. The WBC count was obtained using the XE-2100 automated hematology analyzer (Sysmex). The concordance of $CD34^+$ cell counts between dual- and single-platform methods was confirmed using fresh samples obtained from mobilized blood or apheresed products in our laboratory.

Enrichment of $CD34^+$ cells by using immunomagnetic beads

$CD34^+$ cells were isolated using MACS magnetic cell separation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Two independent isolation experiments were performed by using different methods, that is, direct isolation of CD34-expressing cells (positive selection) and depletion of lineage-positive cells (negative selection). Positive selection was performed using the Indirect CD34 MicroBead Kit (Order no. 130-046-701; Miltenyi Biotec, Bergisch Gladbach, Germany),

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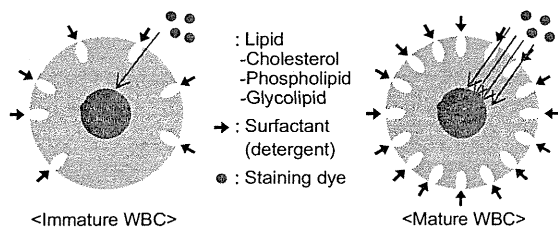

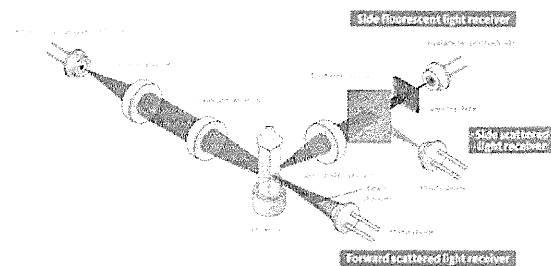

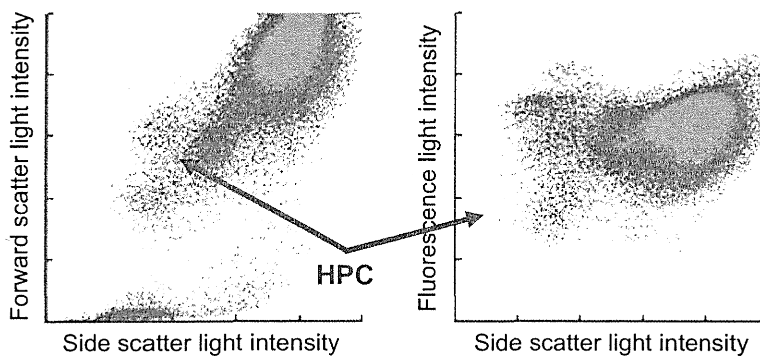

Figure 1. Principle of the HPC enumeration method. (a) The biochemical reaction, including hemolysis and staining with fluorescent dyes, discriminated immature leukocytes because of the differences in the resistance to the surfactant, which was dependent on the lipid contents within the cell membrane. (b) An optical detection method based on flow cytometric analysis was used. (c) The scattergram shows leukocytes as light blue dots and the HPC fraction as purple dots. HPCs were defined by a fixed gate, which was determined from three parameters.

according to the manufacturer's instructions. Briefly, cells were incubated with CD34–hapten–antibodies and an FcR-blocking reagent. After washing with MACS buffer, the cells were incubated with antihapten-conjugated MicroBeads followed by incubation with PE-conjugated anti-CD34 antibodies. After washing, cells were applied onto a column attached to a magnet. The labeled cells, which were trapped in the column within the magnetic field, were retrieved by washing the column after it was removed from the magnet. A Lineage Cell Depletion Kit (Order no. 130-092-211; Miltenyi Biotec, Bergisch Gladbach, Germany) was used for negative selection. This kit consisted of a biotin-conjugated antibody cocktail (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a) and antibiotin-conjugated MicroBeads. Labeling and column separation were performed in the same way as in positive selection. Enriched $CD34^+$ cells were collected as unlabeled cells.

Statistics

Statistical analysis was performed using SPSS 15.0J software (SPSS Inc., Chicago, IL, USA). Correlations were assessed with Pearson's correlation test. Differences between the two groups were assessed by using the nonparametric Wilcoxon signed rank test. *P*-values of <0.05 were considered statistically significant. Differences in the slope of linear regression equations between the two groups were assessed by parallel tests.

RESULTS

Patients and materials

Between 2008 and 2011, a total of 18 healthy donors and 6 patients were enrolled into this study. Seventy-six mobilized blood and apheresis products were used for the analysis. The diagnosis was non-Hodgkin's lymphoma in all patients.

Basal performance of HPC enumeration

The feasibility of the basal performance of HPC enumeration, including reproducibility, sample stability, linearity, and the effects of anticoagulant, was evaluated and confirmed using mobilized blood and apheresis products.

The reproducibility of HPC counts was evaluated by five repeated analyses using five different PB samples (HPC range, 22–59 cells/μL), and the coefficient of variation (CV) was 10.3% (range, 5.6–16.1%). Concerning the sample stability, HPCs were evaluated periodically using independent PB samples at room temperature over 6 h after sampling, and stability was maintained. We also compared HPC counts in PB samples that had been stored for over 24 h with those in fresh samples, and there were no significant differences (data not shown). This suggested that cell viability did not influence HPC counts significantly, at least for 24 h from sampling. The linearity, which was evaluated using diluted PB or apheresis products, was almost concordant with the theoretical values ($R^2 = 0.996$, slope of the regression curve: 0.981). There were no significant differences between apheresis samples with or without EDTA-2K as an anticoagulant.

Correlation between HPCs and CD34$^+$ cells

CD34$^+$ cell counts ranged widely. The median and range in the pre-apheresis PB and intermediate and final apheresis products were 25.9 cells/μL (range, 0.3–453.5 cells/μL), 955.0 cells/μL (range, 379.4–12 830.5 cells/μL), and 597.3 cells/μL (range, 67.1–11 805.3 cells/μL), respectively. There was a very strong correlation between the numbers of HPCs and CD34$^+$ cells in all samples ($R^2 = 0.958$). Strong correlations between these two markers were also observed in both subgroups of the pre-apheresis PB ($R^2 = 0.919$) and apheresis products ($R^2 = 0.958$; Figure 2). There were no statistically significant differences between the slopes of regression equations in PB and apheresis products ($P = 0.729$).

Next, we examined the possibility of predicting the total number of collected CD34$^+$ cells from pre-apheresis PB. From HPCs in pre-apheresis PB, the expected HPC yield was calculated as follows: expected HPC yield = [HPCs in PB (cells/μL)] × [processed blood volume (mL)] × 10^3 × [collection efficiency], where the processed blood volume was 10 L in all apheresis sessions. The expected HPC yield correlated with the number of CD34$^+$ cells in the final products ($R^2 = 0.952$), if the collection efficiency was assumed to be constant (50%). The HPC count in mid-apheresis PBSC products also had a good correlation with the CD34$^+$ cell count in the final product ($R^2 = 0.918$; Figure 3). The slopes of both regression equations on expected HPCs were not significantly different ($P = 0.156$).

Concerning the kinetics of HPCs and CD34$^+$ cells in the PB during mobilization, the changes in HPCs were similar to those in CD34$^+$ cells in all nine patients and donors (Figure 4). The timing of appearance of HPCs in the PB was almost concordant with that of CD34$^+$ cells, which suggested that the appearance of HPCs may be a good indicator for the optimal timing of PBSC collection.

Relationship between HPCs and isolated CD34$^+$ cells

Apheresis products were processed immediately after sampling. To clarify the relationship between HPCs and CD34$^+$ cells, CD34$^+$ cells were isolated from the final products by positive or negative selection using immunomagnetic beads. First, because the attachment of immunomagnetic beads may affect the results of HPC counts, CD34$^+$ cells were enriched by depleting lineage-positive cells. The resulting lineage-negative cells showed two major populations in the HPC scattergram: one population (20.6%) in the HPC area and another in the area corresponding to granulocytes in a representative experiment (Figure 5a). Flow cytometric analysis also showed two populations in the forward *vs.* side scattergram; one population, which accounted for 22.8%, corresponded to CD34$^+$ cells, and the other population comprised cells that were

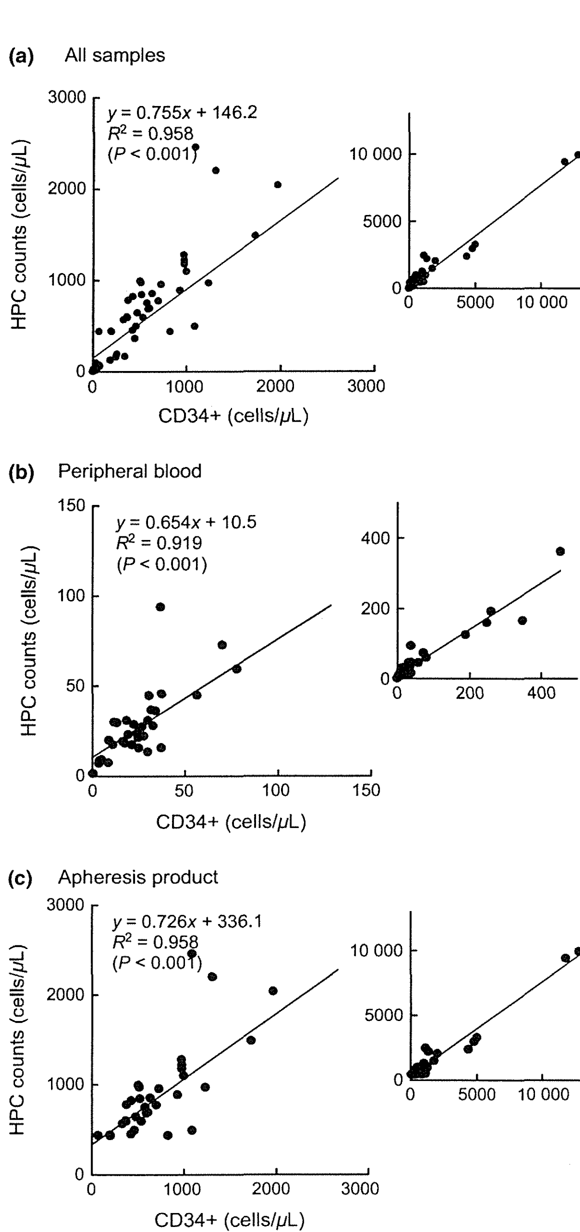

Figure 2. Correlation between HPC counts and the number of $CD34^+$ cells. (a) Samples included the PB and apheresis products (n = 76). Values below 3000 cells/μL are plotted separately (lower left), in addition to all PB samples (upper right). (b) PB samples. Values below 150 cells/μL are plotted separately (lower left), in addition to all PB samples (upper right). (c) Apheresis products. Values below 3000 cells/μL are plotted separately (lower left), in addition to all apheresis samples (upper right).

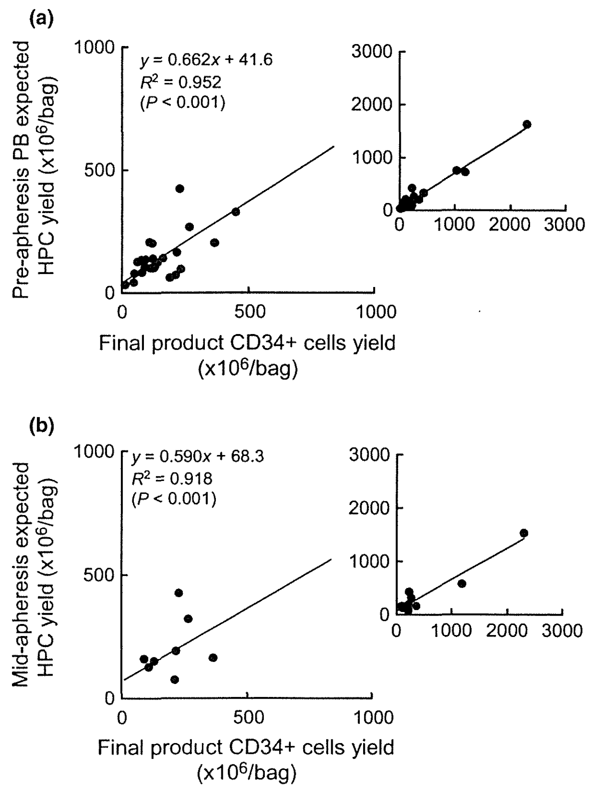

Figure 3. Correlation between expected HPC yield and true $CD34^+$ cell yield. The expected HPC yield was calculated or estimated from HPCs in PB (n = 28) (a) or mid-apheresis products (n = 10) (b). Values below 1000 × 10^6 cells/bag are plotted separately (lower left), in addition to all samples (upper right).

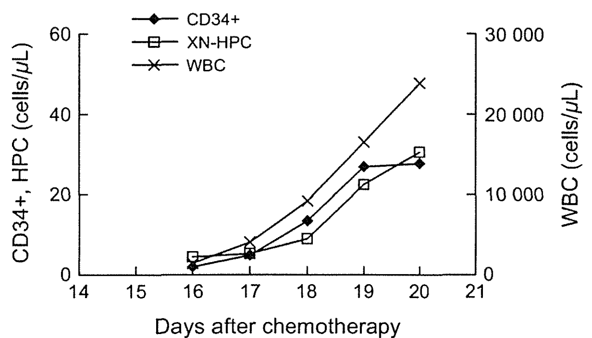

Figure 4. Changes in $CD34^+$ cells, XN-determined HPCs, and WBCs in the PB during the recovery phase from myelosuppression after chemotherapy in a representative patient.

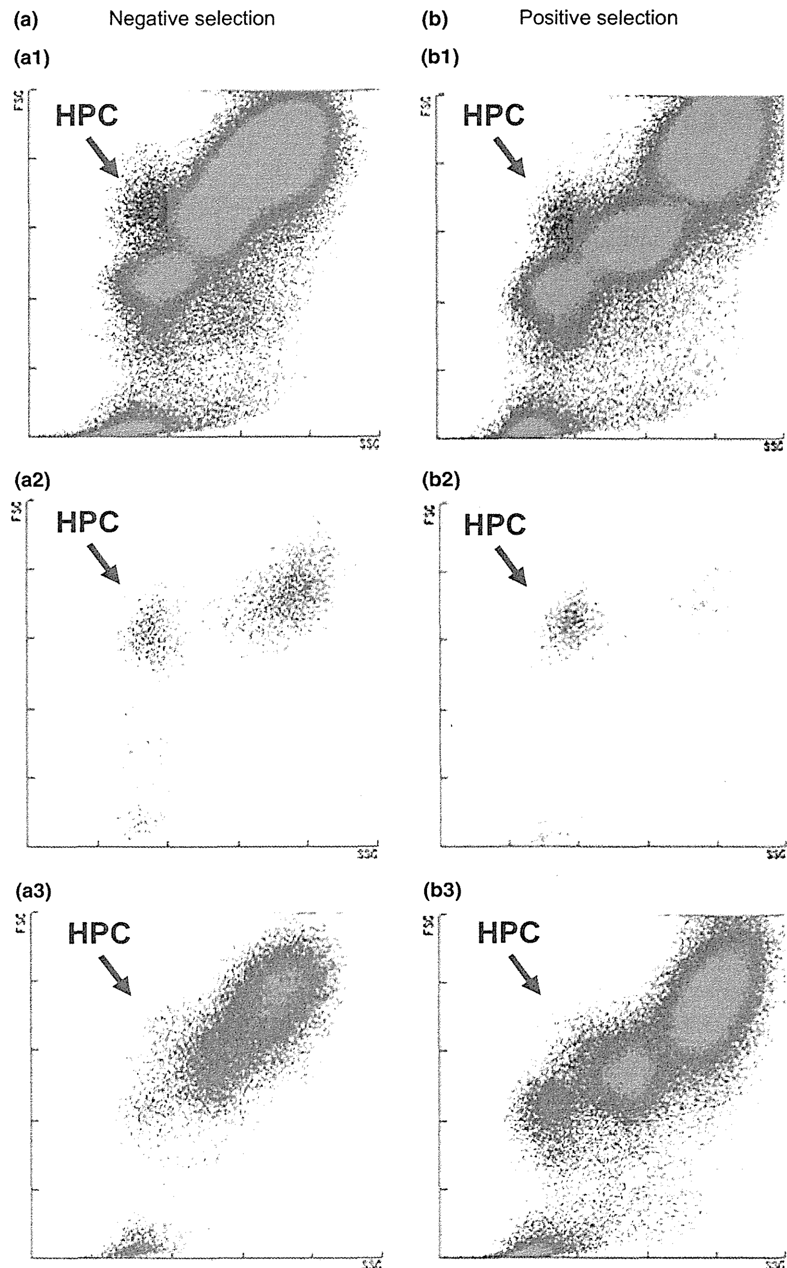

Figure 5. HPC scattergram of immunomagnetically enriched or depleted CD34$^+$ cells in representative experiments. (a) CD34$^+$ cells were enriched from apheresis products by depleting lineage-positive cells (negative selection). (a1) Before separation: CD34$^+$ 0.6%, HPCs 0.7%. (a2) Lineage-negative fraction: CD34$^+$ 22.8%, HPCs 20.6%. (a3) Lineage-positive fraction: CD34$^+$ 0.4%, HPCs 0.5%. (b) CD34$^+$ cells were isolated directly using anti-CD34 antibodies. The location of dots indicated that HPCs may have shifted from their original position. (b1) Before separation: CD34$^+$ 0.5%, HPCs 0.4%. (b2) CD34$^+$ fraction: CD34$^+$ 83.2%, HPCs 70.2%. (b3) CD34-depleted fraction: CD34$^+$ 0.1%, HPCs 0.2%. Dots detected in the HPC area appeared to correspond to CD34$^+$ cell populations, independent of the separation method.

negative for CD34 expression. Microscopic examination of this fraction after cytospin preparation followed by May–Giemsa staining revealed that there were also two major cell populations; these cell populations were mainly composed of promyelocytes (>50%) and blastoid mononuclear cells (approximately 40%). On the other hand, lineage-positive cells, including few CD34$^+$ cells (0.4%), showed few dots in the HPC area (0.5%).

Next, CD34$^+$ cells were directly isolated with anti-CD34 antibodies and analyzed for HPCs. The positively isolated fraction with a CD34$^+$ cell purity of 83.2% showed a single cluster in the HPC area (70.2%) of the scattergram, while the CD34-depleted

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fraction, including 0.1% CD34$^+$ cells, appeared as very few dots in this area (HPCs, 0.2%; Figure 5b).

DISCUSSION

Automated hematology analyzers are usually used in routine laboratory tests. Several authors have reported that the Sysmex SE/XE-series HPC count system is a useful additional function of the automated hematology analyzer [8–22]. However, it has the disadvantage of low counting performance, which is mainly caused by the lack of ability to identify cell populations precisely. In the XN-series model, the performance has been improved by changing the reagent composition and the detection principle. The cell population corresponding to HPCs in the XN-series model is identified by a flow cytometry-based optical detection system that differs from the previous SE- or XE-series hematology analyzers, which utilize the electrical radiofrequency (RF)/direct currency (DC) impedance detection method. The optical detection system allows us to more accurately recognize and differentiate the morphological and biochemical properties of each cell based on the degree of staining. Moreover, the volume of sample required for HPC analysis has been increased from 1 to 4 μL per assay, which has increased the accuracy of HPC, especially at lower concentrations.

In this study, we demonstrated that there was a good correlation between the numbers of HPCs and CD34$^+$ cells in samples from not only the PB but also apheresis products. The correlation coefficient was much closer to 1.0 with this novel XN-determined HPC count than with the previous SE/XE-determined HPC count [8, 9, 13, 16–18, 20, 21]. These results suggested that the number of CD34$^+$ cells in the final products could be predicted from the HPC count in the final products. The correlation was unaffected by the WBC counts, use of EDTA as an anticoagulant, sample types (i.e., PB or PBSC), or timing of collection. Moreover, our data from the experiments using immunomagnetic beads indicated that HPCs reflected the majority of cells in the CD34$^+$ cell fraction. As for the markers of the clinically relevant number of hematopoietic stem cells required for a successful PBSCT, only the numbers of colony-forming cells [31] and CD34$^+$ cells [3, 32–34] have been used so far [2, 35]. Our data suggested the possibility that the number of HPCs may be another candidate marker of hematopoietic stem cells in PBSCT. Secondly, the approximate number of CD34$^+$ cells in the final products may be predicted from the HPC count in pre-apheresis PB. Moreover, the fact that the change in the number of HPCs closely resembled that of CD34$^+$ cells in the PB during mobilization also suggested that HPCs in the PB may be a good indicator for the optimal timing of PBSC collection, as is CD34$^+$ cell concentration [32, 33, 36] and SE/XE-determined HPC counts [9–22]. Thirdly, our data also suggested that the final CD34$^+$ cell yield may be predicted from the HPC count in the mid-apheresis product. This may allow us to adjust the planned total processed blood volume during apheresis so that an optimal and sufficient apheresis session can be accomplished.

Concerning the cell population corresponding to HPCs, we demonstrated in our experiments using immunomagnetic beads that the majority of HPCs overlapped with the CD34$^+$ cell population in the PB or PBSCs. HPCs cannot be retrieved intact because the cell membrane will be damaged or lysed during the assay process. Moreover, any manipulation of cells, whether immunomagnetic beads are used or not, will not allow us to accurately enumerate HPCs because the hemolysis reaction is very sensitive and because the location of the HPC fraction in the scattergram may shift. Because the HPC population was determined by fixed gating in the HPC scattergram of the XN-series automated hematology analyzer, even a subtle shift in the scattergram may result in false HPC values. Therefore, we first evaluated HPCs by using PBSC samples in which CD34$^+$ cells had been enriched by depleting lineage-positive cells without direct attachment to immunomagnetic beads and revealed that the concentrations of CD34$^+$ cells and HPCs were almost equal. Next, the fact that CD34$^+$ cells, which had been isolated using anti-CD34 antibody-conjugated immunomagnetic beads, showed a single cell fraction around the HPC gate in the scattergram, suggesting that most CD34$^+$ cells in this sample fell into the HPC gate. The percentages of HPCs and CD34$^+$ cells may reflect a shift after processing of the cells, including direct attachment to immunomagnetic beads, centrifugation, cell wash. These results demonstrated that the majorities of HPCs and CD34$^+$ cells were almost identical, at least if the sample was derived from mobilized PB or apheresed PBSCs.

Theoretically, in the very primitive stem cell fraction, the number of CD34$^-$ stem cells, whether HPCs are included or excluded, is considered very small and negligible [37–39]. In the fraction of more mature myeloid cells, it is reasonable to speculate that the majority of HPCs exist in the less-differentiated fraction than in the most-differentiated CD34-weakly expressing progenitor cells, which are infrequently observed in the mobilized blood.

Because this method has some advantages, such as rapid assay time, ease, and low cost, it is possible that enumeration of CD34$^+$ cells may be substituted for that of HPCs for determining an optimal stem cell dose for PBSCT. Before the era of CD34$^+$ cell enumeration, the number of granulocyte–macrophage colony-forming cells (GM-CFCs) was the main marker for PBSCT [29]. Although there is a good correlation between the numbers of GM-CFCs and CD34$^+$ cells, there is an almost 10-fold difference in quantity; in contrast, the number of HPCs is more similar to that of CD34$^+$ cells. Moreover, the use of fixed gating and the operability on an automated hematology analyzer will facilitate the standardization of HPC enumeration protocols.

In conclusion, we developed a novel HPC enumeration method, carried out without using monoclonal antibodies on an XN-series automated hematology analyzer. The final amount of collected CD34$^+$ cells may be predicted from the total number of HPCs in the final products, as well as from pre-apheresis PB and intermediate products during apheresis. HPCs may also be a good indicator for the optimal timing of PBSC collection. Because the number of patients in this study was small, interpretation of the results is limited. Therefore, we are currently conducting a multicenter prospective study, in which a larger number of patients and healthy donors will be enrolled, to determine the usefulness of HPCs in practice.

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RT, TK, AY, and SO designed the research project, carried out the experiments, and analyzed the data. YH contributed to the data analysis and organization of the study. AN, NT, and SY helped with sampling. RT, AY, and SO wrote the manuscript. All the authors interpreted data, reviewed the manuscript, and approved the final version. This study was conducted in collaboration with Sysmex Corporation, Kobe, Japan.

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ORIGINAL ARTICLE

Positive impact of chronic graft-versus-host disease on the outcome of patients with *de novo* myelodysplastic syndrome after allogeneic hematopoietic cell transplantation: a single-center analysis of 115 patients

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Abstract

To evaluate the impact of graft-versus-host disease (GVHD) and prognostic factors for patients with myelodysplastic syndrome (MDS) after allogeneic hematopoietic cell transplantation (allo-HCT), we retrospectively reviewed 115 patients with MDS or acute myeloid leukemia with multilineage dysplasia (AML-MLD) after allo-HCT at our center. Eighty one patients received reduced-intensity conditioning (RIC) regimens, whereas 34 received myeloablative conditioning regimens. Although the RIC group was significantly older and included more patients with poor cytogenetic risk, no difference in 4-yr overall survival (OS) was seen between the two groups. In a multivariate analysis, covariates associated with a worse OS were the French-American-British stage of refractory anemia excess blasts in transformation/ AML-MLD at peak, poor cytogenetic risk, bone marrow blasts of 20% or higher at HCT and the absence of chronic GVHD (cGVHD). By using semi-landmark analyses, we found that the presence of cGVHD significantly improved OS in high-risk patients or the RIC group. However, there was no difference in OS between those with and without cGVHD among low-risk MDS patients. These findings suggest that the graft-versus-leukemia effect may be more beneficial in high-risk patients who do not receive intensive preparative regimens.

Key words myelodysplastic syndrome; allogeneic hematopoietic cell transplantation; graft-versus-host disease; graft-versus-leukemia effect

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Allogeneic hematopoietic cell transplantation (allo-HCT) has been assumed to be the only treatment modality with curative potential for patients with myelodysplastic syndrome (MDS). However, about 90% of MDS cases occur in elderly patients above the age of 60 yrs (1) and a substantial proportion of them are more likely to have a worse performance status and an increased comorbidity. As a result, myeloablative conditioning (MAC) regimens are less commonly used for patients with MDS because of an increased risk of non-relapse mortality (NRM). However, some studies have reported that the dose intensity of the conditioning regimen plays an important role in controlling the disease after allo-HCT for MDS or acute myeloid leukemia (AML) (2, 3). Reduced-intensity conditioning regimens (RIC) have been developed to decrease the risk of NRM with less-intensive conditioning for elderly or less-fit patients while preserving a graft-versus-leukemia (GVL) effect by an alloimmune reaction as an antitumor effect (4, 5). The European Group for Blood and Marrow Transplantation reported that, among patients with MDS who underwent allo-HCT from a sibling donor, the RIC group was associated with a lower incidence of NRM and a higher risk of relapse in comparison with the

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MAC group, whereas overall survival (OS) was similar in both groups (6).

Although an alloimmune reaction by donor T-cells is important for disease control after allo-HCT, especially in the RIC setting, the significance of this effect has not been well documented in patients with MDS. Therefore, we retrospectively reviewed the medical records of 115 patients with *de novo* MDS or AML with multilineage dysplasia (AML-MLD) who underwent their first allo-HCT at our center, and evaluated the impact of graft-versus-host disease (GVHD) and prognostic factors for the outcome in patients with MDS after allo-HCT.

Patients and methods

Patients

This study included patients with *de novo* MDS or AML-MLD who underwent their first allo-HCT at our center between January 2000 and December 2009. The study protocol was reviewed and approved by the institutional ethics committee. Therapy-related MDS and cord blood transplant recipients were excluded. Therapy-related MDS was defined as disease arising in patients who were treated with irradiation, chemotherapy, or both for hematologic malignancies or other cancers. Disease stages were categorized according to the French-American-British (FAB) classification (7). AML-MLD was defined as AML with more than 30% bone marrow (BM) myeloblasts and morphological features of myelodysplasia, or a prior history of MDS. Patients with MDS were classified into two diagnostic groups (Low/Intermediate-1 and Intermediate-2/High) at diagnosis and at peak according to the International Prognostic Scoring System (IPSS) (8). Cytogenetic risk groups were determined according to IPSS using the cytogenetic information at diagnosis. Matching between the donor and recipient was determined according to donor–recipient HLA-A, HLA-B, and HLA-DR compatibility.

Myeloablative conditioning regimens included cyclophosphamide (Cy, 60 mg/kg for 2 d) plus busulfan (Bu, orally 4 mg/kg for 4 d or i.v. 3.2 mg/kg for 4 d) (Bu/Cy) or total body irradiation (TBI, 12 Gy) (TBI/Cy). RIC regimens included Bu (orally 4 mg/kg for 2 d or i.v. 3.2 mg/kg for 2 d) plus fludarabine (Flu, 30 mg/m^2 for 6 d) (Flu/Bu) or cladribine (2-CdA, 0.11 mg/kg for 6 d) (2-CdA/Bu). In a subset of patients who received RIC, low-dose TBI (2 or 4 Gy) and/or low-dose antithymocyte globulin (ATG) (total dose 5–10 mg/kg Fresenius or 2.5–5 mg/kg Thymoglobulin) were added. GVHD prophylaxis included either cyclosporine or tacrolimus alone or a combination of either of the calcineurin inhibitors and methotrexate. The decision regarding the intensity of the conditioning regimen and GVHD prophylaxis for each patient was made at the discretion of the attending physicians based on a review of the patient's age, disease status, comorbidities, performance status and HLA compatibility.

Neutrophil and platelet engraftment dates were defined as the first of three consecutive days with an absolute neutrophil count of 0.5×10^9/L or higher and an untransfused platelet count of 2.0×10^9/L or higher. Acute and chronic GVHD (cGVHD) were diagnosed and graded according to standard criteria (9). Response and relapse of the disease were defined according to standard hematologic criteria.

Statistical analysis

We used the Chi-square analysis and Fisher's exact test to compare categorical covariates and the Mann–Whitney U test to compare continuous covariates. OS was estimated by the Kaplan–Meier method, and differences between groups were evaluated by the log-rank test. Relapse and NRM were considered as competing risk events for each other. The probabilities of relapse and NRM were estimated by the cumulative incidence functions, and differences between groups were evaluated by the Gray test (10, 11). OS and the incidences of relapse and NRM were estimated as probabilities at 4 yrs from allo-HCT. To evaluate the effect of cGVHD on OS, we performed semi-landmark analyses (12). For patients with cGVHD, OS was estimated as the probability from the onset of cGVHD by the Kaplan–Meier method. A landmark comparison group consisted of survivors without cGVHD at day 138 (landmark day), which was the median time of the onset of cGVHD with OS for this group estimated as the probability from the landmark day. The Cox proportional hazards regression model was used for univariate and multivariate analyses, and a hazard ratio was calculated in conjunction with a 95% confidence interval (CI). For the assumption of proportional hazards over time, acute GVHD (aGVHD) and cGVHD were treated as time-dependent covariates (13). For multivariate analyses, we decided to include covariates with a P-value of <0.1 in univariate analyses. In addition, we included conditioning regimens and GVHD in these models to evaluate their effects on the outcome. The statistical analysis was performed with R-Project (version 2.2.1; http://www.r-project.org/).

Results

Patient characteristics

The characteristics of a total of 115 patients are summarized in Table 1. The median age was 55 yrs (range: 19–68) and the median follow-up of surviving patients was 40 months (range: 4–130). Eighty one patients (70%) received RIC regimens, whereas 34 (30%) received MAC regimens. According to the FAB stage at peak, the proportions of patients with refractory anemia (RA)/refractory anemia with ringed sideroblasts (RARS), refractory anemia

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Table 1 Patient characteristics

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Period of HCT (%)			
2000–2004	71 (62)	18 (53)	53 (65)
2005–2009	44 (38)	16 (47)	28 (35)
Age at HCT, median (range)	55 (19–68)	46 (23–57)	57 (19–68)
Age at HCT, yrs			
≧50 yrs (%)	84 (73)	10 (29)	74 (91)
Patient sex, male (%)	82 (71)	24 (71)	58 (72)
FAB stage at diagnosis (%)			
RA/RARS	45 (39)	13 (38)	32 (40)
RAEB/CMMoL	44 (38)	12 (36)	32 (40)
RAEB-T/AML-MLD	26 (23)	9 (26)	17 (20)
IPSS at diagnosis (%)			
Low/Intermediate-1	37 (32)	13 (38)	24 (30)
Intermediate-2/High	64 (56)	16 (47)	48 (59)
Unknown	14 (12)	5 (15)	9 (11)
FAB stage at peak (%)			
RA/RARS	22 (19)	6 (18)	16 (20)
RAEB/CMMoL	38 (33)	10 (29)	28 (34)
RAEB-T/AML-MLD	55 (48)	18 (53)	37 (46)
IPSS at peak (%)			
Low/Intermediate-1	24 (21)	6 (18)	18 (22)
Intermediate-2/High	77 (67)	23 (68)	54 (67)
Unknown	14 (12)	5 (14)	9 (11)
Cytogenetic risk group (%)			
Good/Intermediate	75 (65)	27 (79)	48 (59)
Poor	40 (35)	7 (21)	33 (41)
BM blasts at HCT, median (range)	5 (0–78)	3 (0–46)	4 (0–78)
≦4%	60 (52)	18 (53)	42 (52)
5–19%	38 (33)	10 (29)	28 (35)
≧20%	10 (9)	3 (9)	7 (8)
Unknown	7 (6)	3 (9)	4 (5)
Disease duration, months, median (range)	9 (1–200)	8 (2–200)	10 (1–172)
Karnofsky score at HCT (%)			
90–100	96 (83)	29 (85)	67 (83)
Transfusion dependence (%)	89 (77)	27 (79)	62 (77)
Prior chemotherapy (%)	68 (59)	22 (65)	46 (57)
Donor (%)			
Related	55 (48)	12 (35)	43 (53)
Unrelated	60 (52)	22 (65)	38 (47)
HLA matching (%)			
HLA match (6/6)	101 (88)	31 (91)	70 (86)
HLA mismatch (5/6)	14 (12)	3 (9)	11 (14)
Source of stem cells (%)			
Peripheral blood	52 (45)	11 (32)	41 (51)
BM	63 (55)	23 (68)	40 (49)
Sex mismatch (%)			
Female donor/Male recipient	36 (31)	13 (38)	23 (28)
Other combination	79 (69)	21 (62)	58 (72)
Follow-up duration for survivors, months, median (range)	40 (4–130)	40 (4–130)	47 (4–125)

(continued)

Table 1. (contiuned)

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Conditioning regimen			
MAC (%)			
CY/TBI		15 (44)	
Bu/CY		19 (56)	
Reduced intensity conditioning			
Flu/Bu-based			65 (80)
2-CdA/Bu-based			16 (20)
TBI-containing			23 (28)
ATG-containing			26 (32)
GVHD prophylaxis (%)			
CSP			26 (32)
CSP+MTX		24 (71)	37 (46)
TAC			2 (2)
TAC+MTX		10 (29)	16 (20)

MAC, myeloablative conditioning; RIC, reduced intensity conditioning; HCT, allogeneic hematopoietic cell transplantation; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; CMMoL, chronic myelomonocytic leukemia; RAEB-T, refractory anemia with excess blasts in transformation; AML-MLD, acute myeloid leukemia with multilineage dysplasia; BM, bone marrow; mons, months; CY, cyclophosphamide; TBI, total body irradiation; Bu, busulfan; ATG, antithymocyte globulin; Flu, fludarabine; 2-CdA, cladribine; CSP, cyclosporine; MTX, methotrexate; TAC, tacrolimus; GVHD, graft-versus-host disease; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome.

with excess blasts (RAEB)/chronic myelomonocytic leukemia (CMMoL), and refractory anemia excess blasts in transformation (RAEB-T)/AML-MLD were 19%, 33%, and 48%, respectively. According to the cytogenetic risk at diagnosis, the proportions of patients with good/intermediate and poor risk were 65% and 35%, respectively. According to the IPSS risk at peak, the proportions of patients with Low/Intermediate-1 and Intermediate-2/High were 21% and 67%, respectively, and 12% of the patients did not have evaluable data. BM blast counts at allo-HCT were 4% or less in 52%, 5–19% in 33%, 20% or higher in 9%, and not evaluable in 6%. The RIC group was significantly older than the MAC group (median, 57 vs. 46 yrs, $P < 0.001$) and included more patients with poor cytogenetic risk (41% vs. 21%, $P = 0.03$).

Conditioning regimen and GVHD prophylaxis

The conditioning regimen and GVHD prophylaxis are shown in Table 1. The MAC group included either Bu/CY or TBI/CY, followed by a combination of methotrexate and tacrolimus or cyclosporine. The RIC group included Flu/Bu or 2-CdA/Bu, followed by either cyclosporine or tacrolimus alone or a combination of either of the calcineurin inhibitors and methotrexate.

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Hematopoietic recovery

A total of 113 patients achieved primary engraftment with a median time to reach a neutrophil count of 0.5×10^9/L or higher and a platelet count of 2.0×10^9/L or higher of 14 d (range, 10–40 d) and 22 d (range, 8–105 d), respectively. The median times to reach these neutrophil and platelet counts were earlier in the RIC group than the MAC group (neutrophil: 14 vs. 19 d, $P < 0.001$; platelet: 21 vs. 29 d, $P = 0.005$), as shown in Table 2. None of the patients experienced primary graft failure. All but two patients, who died before day 30 after allo-HCT without evidence of engraftment, were assessed for hematopoietic recovery, and 6 (5%) experienced secondary graft failure.

Graft-versus-host disease

The 113 patients who achieved engraftment was evaluated for aGVHD. The incidence of grade II–IV aGVHD was 42% and that of grade III–IV aGVHD was 14%, as shown in Table 2. There was no significant difference between the RIC and MAC groups in the incidence of aGVHD. Among the 107 patients who survived more than 100 d after allo-HCT, 10 (9%) developed limited cGVHD and 48 (45%) developed extensive cGVHD. There was no significant difference between the RIC and MAC groups with regard to the incidence of cGVHD.

Non-relapse mortality

The 4-yr incidence of NRM was 29% in the MAC group and 33% in the RIC group ($P = 0.89$) (Fig. 1A). In a univariate analysis, covariates associated with a higher incidence of NRM were recipient sex [female, hazard ratio (HR) 2.9, 95% CI 1.1–7.5, $P = 0.03$], IPSS risk at diagnosis (Int-2/High, HR 2.2, 95% CI 1.1–4.7, $P = 0.04$), the FAB stage at peak (RAEB/CMMoL, HR 2.8, 95% CI 1.0–7.7, $P = 0.05$), cytogenetic risk at diagnosis (poor, HR 2.0, 95% CI 1.1–4.0, $P = 0.03$), BM blasts at HCT (20% or higher, HR 4.1, 95% CI 1.7–10.2, $P = 0.002$), and the presence of aGVHD (grade III–IV, HR 4.4, 95% CI 2.2–9.0, $P < 0.001$), as shown in Table S1. In a multivariate analysis (Table 3), the covariates associated with a higher incidence of NRM were the presence of aGVHD (grade III–IV, HR 6.9, 95% CI 2.7–17.4, $P < 0.001$) and BM blasts at HCT (20% or higher, HR 3.6, 95% CI 1.3–9.9, $P = 0.01$). cGVHD in this model was not an independent factor for NRM when substituted for grade III–IV aGVHD (data not shown).

Relapse

The 4-yr incidence of relapse was 26% in the MAC group and 25% in the RIC group ($P = 0.97$) (Fig. 1B). In a univariate analysis, the only covariate associated with a higher relapse rate was prior chemotherapy (HR 2.5, 95% CI 1.1–5.8, $P = 0.04$), as shown in Table S1. In a multivariate analysis (Table 3), covariates associated with a higher relapse rate were prior chemotherapy (HR 4.3, 95% CI 1.2–15.9, $P = 0.03$), BM blasts at HCT (5–19%, HR 4.3, 95% CI 1.5–12.8, $P = 0.008$) and the absence of cGVHD (HR 12.7, 95% CI 3.1–52.6, $P < 0.001$). Grade II–IV or III–IV aGVHD in this model was not an independent factor for relapse when substituted for cGVHD (data not shown).

Table 2 Transplantation outcome

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Graft failure (%)			
Primary	0 (0)	0 (0)	0 (0)
Secondary	6 (5)	1 (3)	5 (6)
Engraftment			
Neutrophils ≧ 0.5×10^9/L Median days (range)	14 (10–40)	19 (10–40)	14 (10–27)
Platelets ≧ 20×10^9/L Median days (range)	22 (8–105)	29 (13–90)	21 (8–105)
Acute GVHD (%)			
II–IV	48 (42)	12 (35)	36 (44)
III–IV	16 (14)	4 (11)	12 (15)
Onset, median days (range)	30 (5–98)	34 (9–66)	31 (9–68)
Chronic GVHD (%)			
Limited	10 (10)	4 (14)	6 (8)
Extensive	48 (47)	11 (39)	37 (50)
Onset, median days (range)	138 (100–1090)	124 (100–245)	134 (100–1090)

MAC, myeloablative conditioning; RIC, reduced intensity conditioning; GVHD, graft-versus-host disease.

Overall survival

In the overall population, the 4-yr OS was 44%. Although patients in the RIC group were older and had a worse cytogenetic risk, no difference in OS was seen between the two groups (47% in the MAC group vs. 42% in the RIC group, $P = 0.84$) (Fig. 1C). Fifty two patients (45%) were alive and 63 (55%) had died. Disease relapse or progression (40%) was the most common cause of death, followed by non-relapse causes complicated by organ failure (23%), infection (19%), GVHD (6%), and others (12%) (Table 4). In a univariate analysis, covariates associated with a worse OS were older age (60 yrs or older, HR 1.7, 95% CI 1.0–2.9, $P = 0.04$), the FAB stage at diagnosis (RAEB/CMMoL, HR 1.8, 95% CI 1.0–3.2, $P = 0.04$), IPSS risk at diagnosis (Int-2/High, HR 2.4, 95% CI 1.3–4.4, $P < 0.001$), the FAB stage at peak (RAEB/CMMoL, HR 2.3, 95% CI 1.0–5.2, $P = 0.04$ RAEB-T/AML-MLD, HR 2.6, 95% CI 1.2–5.7,

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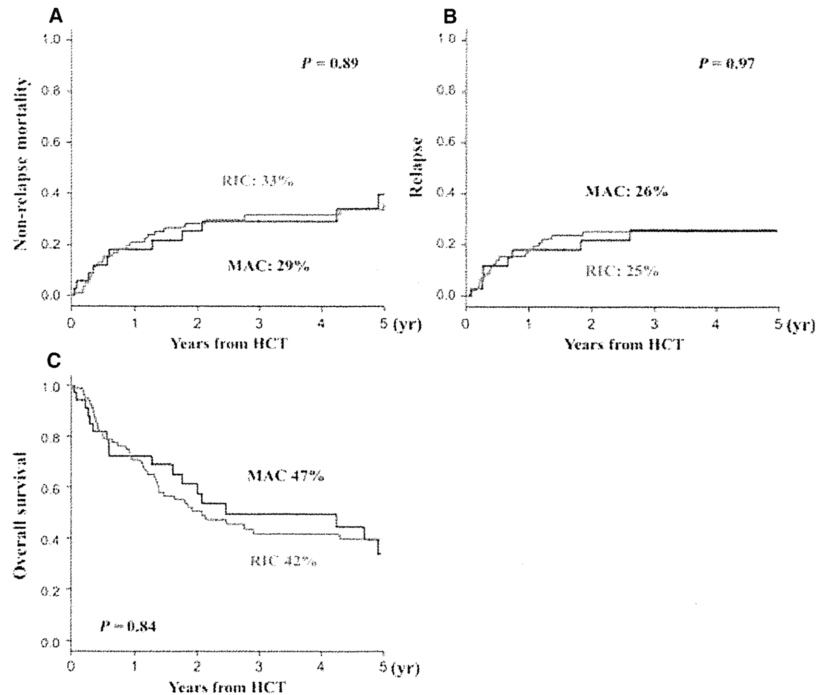

Figure 1 Outcomes stratified according to the intensity of the conditioning regimens. non-relapse mortality (A), Relapse (B) and overall survival (C) of patients with myelodysplastic syndrome receiving allo-hematopoietic cell transplantation after myeloablative conditioning or reduced-intensity conditioning regimens.

$P = 0.01$), IPSS risk at peak (Int-2/High, HR 2.3, 95% CI 1.1–5.0, $P = 0.02$), cytogenetic risk at diagnosis (poor, HR 2.2, 95% CI 1.3–3.7, $P < 0.001$), BM blasts at HCT (20% or higher, HR 3.4, 95% CI 1.6–7.2, $P < 0.001$), and the presence of aGVHD (Grade III–IV, HR 2.8, 95% CI 1.5–5.4, $P = 0.001$), as shown in Table S1. In a multivariate analysis (Table 3), covariates associated with a worse OS were the FAB stage at peak (RAEB-T/AML-MLD, HR 3.3, 95% CI 1.2–8.6, $P = 0.02$), cytogenetic risk at diagnosis (poor, HR 2.1, 95% CI 1.1–6.9, $P = 0.01$), BM blasts at HCT (20% or higher, HR 3.0, 95% CI 1.3–6.9, $P = 0.01$) and the absence of cGVHD (HR 2.0, 95% CI 1.1–4.0, $P = 0.04$). The presence of grade III–IV aGVHD was significantly associated with a worse OS (HR 5.4, 95% CI 2.5–11.4, $P < 0.001$) when this was substituted for cGVHD in this model.

In semi-landmark analyses for the entire population, the OS of patients with cGVHD tended to be better than that of patients without cGVHD ($P = 0.11$) (Fig. 2A). When the analysis was limited to the RIC group, the OS of patients with cGVHD was significantly better than that of patients without cGVHD ($P = 0.005$) (Fig. 2B). We also found that, in patients with poor cytogenetic risk, the OS of patients with cGVHD was significantly better than that of patients without cGVHD ($P = 0.003$) (Fig. 2C), whereas in patients with good/intermediate cytogenetic risk, there was no significant difference in OS between the two groups ($P = 0.76$) (Fig. 2D). In patients with BM blasts 5% or higher at HCT, the OS of patients with cGVHD was significantly better than that of patients without cGVHD ($P = 0.02$) (Fig. S1A), whereas in patients with BM blasts <5% at HCT, there was no significant difference in OS between the two groups ($P = 0.59$) (Fig. S1B).

Impact of extensive cGVHD in the RIC group

The median age in the RIC group was 57 (19–68) yrs. Among the 81 patients in the RIC group, 46 patients (58%) had cGVHD. The majority (86%) of patients with cGVHD developed extensive cGVHD. We also conducted a multivariate analysis limited to the patients pre-treated with RIC (Table S2) and found that the absence of extensive cGVHD was significantly associated with a worse OS (HR 2.4, 95% CI 1.2–5.5, $P = 0.001$) and a higher relapse rate (HR 13.1, 95% CI 4.0–43.9, $P < 0.001$). The presence of extensive cGVHD in this model was not an independent factor for NRM (HR 0.9, 95% CI 0.3–2.7, $P = 0.85$) when substituted for Grade III–IV aGVHD.

Discussion

We performed retrospective analyses of 115 patients with *de novo* MDS or AML-MLD who received their first allo-HCT at our center. By multivariate analyses, we found that the presence of cGVHD significantly reduced relapse and improved OS. To evaluate these results, we considered GVHD to be a time-dependent covariate and analyzed data from all patients to avoid bias from not considering patients

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Table 3 Multivariate analysis for NRM, relapse, and OS

	NRM		Relapse		OS	
Variable	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age						
<60 yrs			1	0.72	1	0.33
≧60 yrs			1.2 (0.5–3.2)		1.4 (0.7–2.6)	
Prior chemotherapy						
No			1	0.03		
Yes			4.3 (1.2–15.9)			
Conditioning regimens						
MAC	1	0.33	1	0.77	1	0.63
RIC	0.7 (0.3–1.5)		0.9 (0.3–2.6)		1.2 (0.6–2.5)	
FAB stage at peak						
RA/RARS	1		1		1	
RAEB/CMMoL	1.2 (0.5–2.7)	0.68	0.6 (0.1–4.8)	0.57	1.9 (0.6–5.9)	0.28
RAEB-T/AML-MLD	2.3 (0.7–7.3)	0.14	0.7 (0.1–4.8)	0.73	3.3 (1.2–8.6)	0.02
Cytogenetic risk group						
Good/Intermediate	1	0.68	1	0.04	1	0.01
Poor	1.2 (0.5–2.7)		2.7 (1.1–6.9)		2.1 (1.1–6.9)	
BM blasts at HCT						
≦4%	1		1		1	
5–19%	1.2 (0.5–2.9)	0.75	4.3 (1.5–12.8)	0.008	1.6 (0.7–3.4)	0.28
≧20%	3.6 (1.3–9.9)	0.01	4.6 (0.9–23.4)	0.07	3.0 (1.3–6.9)	0.01
GVHD						
Grade III–IV aGVHD						
No	1	<0.001				
Yes	6.9 (2.7–17.4)					
cGVHD						
Yes			1	<0.001	1	0.04
No			12.7 (3.1–52.6)		2.0 (1.1–4.0)	

NRM, non-relapse mortality; OS, overall survival; HCT, allogeneic hematopoietic cell transplantation; HR, hazard ratio; CI, confidence interval; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; CMMoL, chronic myelomonocytic leukemia; RAEB-T, refractory anemia with excess blasts in transformation; AML-MLD, acute myeloid leukemia with multilineage dysplasia; BM, bone marrow; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease.

Covariates examined for NRM; Period of HCT, Patient sex, Conditioning regimens, FAB stage at peak, Cytogenetic risk group, BM blast at HCT, The presence of Grade III–IV aGVHD. Covariates examined for Relapse rate; Period of HCT, Age, Patient sex, Prior chemotherapy, Conditioning regimens, FAB stage at peak, Cytogenetic risk group, BM blast at HCT, The presence of cGVHD. Covariates examined for OS; Period of HCT, Conditioning regimens, FAB stage at peak, Cytogenetic risk group, BM blast at HCT, The presence of cGVHD.

who died or relapsed too early to develop acute or chronic GVHD. Some studies that used the same statistical method reported that cGVHD had beneficial effects on relapse in patients receiving allo-HCT after MAC (14, 15). In addition, others showed that the presence of cGVHD was an independent factor in reducing relapse and improving progression-free survival (PFS) in the setting of non-MAC regimens (12) or RIC regimens (16). Similar to our study, Valcárcel *et al.* (16) demonstrated that the development of cGVHD was the strongest factor in reducing relapse and improving survival in patients with high-risk MDS and AML receiving allo-HCT after RIC.

There has been no previous study on the effect of cGVHD on OS according to the conditioning regimen and disease status at allo-HCT. To clarify these questions, we used semi-landmark analyses to evaluate the effect of cGVHD on OS in various subgroups. In the current study, the presence of cGVHD predominantly improved OS in the setting of RIC, but did not affect OS in the MAC group (data not shown). In addition, the presence of cGVHD was significantly associated with the improvement in OS in high-risk patients with BM blasts of 5% or higher at allo-HCT or poor cytogenetic risk, whereas it did not affect OS in low-risk patients. These findings suggest that the benefit of the GVL effect appeared to be more prominent in patients with high-risk MDS who did not receive intensive preparative regimens.

Our findings may suggest that extensive cGVHD is beneficial for patients pre-treated with RIC because of elderly age or less-fit conditions. Valcárcel *et al.* reported that cGVHD was significantly associated with reducing relapse and improving OS without increasing NRM in high-risk AML and MDS patients pre-treated with RIC. In their study,

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Table 4 Cause of death

No. of patients	All $N = 115$	MAC $N = 34$	RIC $N = 81$
Cause of death			
All Causes (% of all patients)	63 (55)	18 (53)	45 (56)
Progression (% of all death)	25 (40)	7 (39)	18 (40)
Organ failure (%)	14 (23)	5 (28)	9 (20)
Multiple organ failure	3	1	2
Veno-occlusive disease	3	1	0
Renal failure	1	0	1
Cardiac failure	1	1	0
Diffuse alveolar hemorrhage	7	2	5
Infection (%)	12 (19)	3 (17)	9 (20)
Bacterium	7	2	5
Fungus	3	0	3
Virus	2	1	1
Bleeding (%)	2 (3)	0 (0)	2 (4)
Secondary cancer (%)	4 (6)	0 (0)	4 (10)
GVHD (%)	4 (6)	2 (11)	2 (4)
Unknown (%)	2 (3)	1 (5)	1 (2)

MAC, myeloablative conditioning; RIC, reduced intensity conditioning; GVHD, graft-versus-host disease.

the cumulative incidence of cGVHD was 53% and extensive cGVHD accounted for the majority (94%) of that (16). Baron *et al.* (12) showed a comparable incidence of extensive cGVHD and reported the same results in AML and MDS patients with extensive cGVHD pre-treated with non-MAC regimens.

It is difficult to induce cGVHD 'moderately' on purpose, and the induction of cGVHD may lead to an increased risk of NRM. When we wish for the presence of cGVHD without a devastating outcome, there are two possible choices. First, G-CSF-mobilized peripheral blood mononuclear cells (G-PBMC) may be a preferable stem cell source when compared with BM. Some studies have shown that the use of G-PBMC as a stem cell source increased the frequency of cGVHD with comparable survival as compared with BM (17–19). Second, GVHD prophylaxis without ATG may be another beneficial option, as ATG has been shown to significantly decrease the incidence of cGVHD (20–22).

As the major causes of treatment failure were disease relapse and progression, treatment strategies before or after allo-HCT to reduce the risk of relapse remain a significant consideration for patients with high-risk MDS. The use of some additional treatment might be effective, especially for patients with high-risk MDS without cGVHD. Azacitidine is a DNA hypomethylating agent to show a significantly prolonged OS compared with conventional care regimens in patients with intermediate-2 and high-risk MDS (23, 24). The use of low-dose azacitidine as pre-emptive and maintenance treatment may prolong survival in patients with higher-risk MDS or AML after allo-HCT (25–27). Azacitidine also appears to induce leukemic cell differentiation and increase the expression of human leukemic antigen DR-1 (HLA-DR) and several tumor-associated antigens that could potentially enhance the GVL effect (28–30). We were not

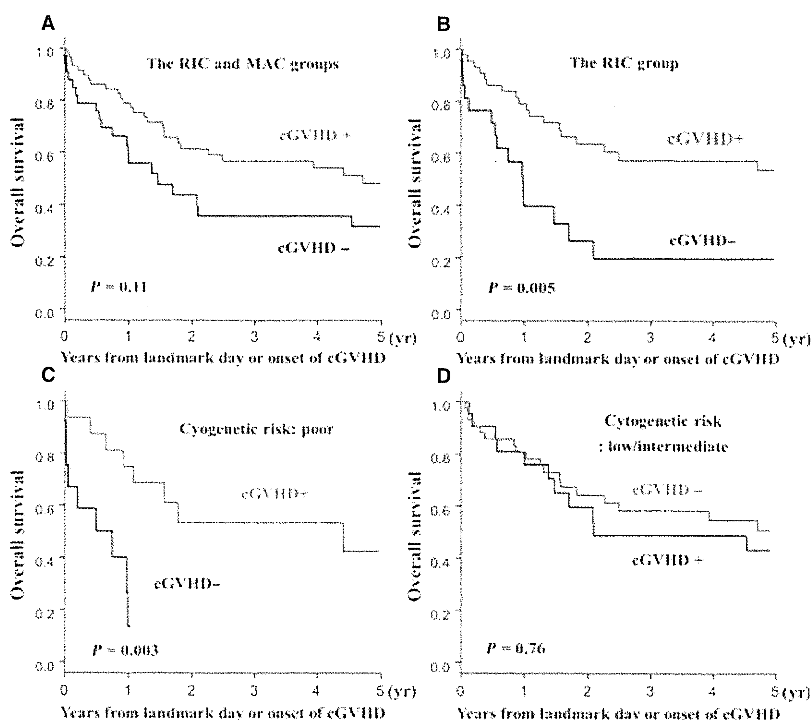

Figure 2 Semilandmark plots illustrating the impact of chronic graft-versus-host disease (GVHD) on overall survival (OS) of patients with myelodysplastic syndrome receiving allo-hematopoietic cell transplantation. OS curves of patients with or without chronic GVHD are shown for the entire population (A), the reduced-intensity conditioning group (B), patients with poor cytogenetic risk (C), and patients with low/intermediate cytogenetic risk (D).

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able to assess the effect of Azacitidine before or after allo-HCT in patients with MDS, because patients who received Azacitidine were not included in our study. These issues need to be addressed in a prospective study.

We also analyzed the impact of aGVHD on outcomes after allo-HCT. The presence of grade II–IV aGVHD did not significantly influence the outcome. On the other hand, the presence of grade III–IV aGVHD was significantly associated with a worse OS and a higher incidence of NRM. Several studies have analyzed the effect of aGVHD on the prognosis after allo-HCT, but only a few have shown that aGVHD has a positive impact (12, 15, 16, 31). Kanda *et al.* (31) reported that grade I aGVHD had a beneficial effect on PFS in high-risk patients. However, we were not able to evaluate the effect of grade I aGVHD because of the small number of patients.

In the present study, OS, relapse and NRM did not differ significantly between the MAC and RIC groups, although the RIC group had significantly higher proportions of elderly patients and those with poor cytogenetic risk. Several previous studies have analyzed MDS and AML patients who received allo-HCT after MAC or RIC regimens (2, 6, 32, 33). In some studies, OS and PFS tended to be similar between the MAC and RIC groups, with a decreased incidence of NRM offset by an increased incidence of relapse in the RIC group. In other studies, there were no differences in relapse or NRM between the MAC and RIC groups, with a comparable OS (34, 35), and our results were consistent with the latter results.

The other major covariates that influenced OS in the present study were poor cytogenetic risk at diagnosis and the disease status at allo-HCT. Poor cytogenetic risk was also a significant factor for the increased risk of relapse, which was consistent with previous reports (32, 33, 36, 37). Although some studies have reported that a low pre-transplant tumor burden was essential for the success of allo-HCT in patients with MDS (35, 38, 39), it remains to be determined whether induction chemotherapy should be given to reduce the tumor burden before allo-HCT. Previous studies have shown that chemotherapy prior to allo-HCT did not improve OS because of the possibility of an increased incidence of NRM (38–40). In the present study, prior chemotherapy was significantly associated with an increased risk of relapse, but did not affect OS or NRM. This result may be explained by the fact that patients who need chemotherapy prior to HCT are probably those with high-risk disease.

Our study has several limitations, and thus the results must be interpreted with caution. These limitations include the retrospective nature of the study including the fact that therapeutic strategies were chosen at the discretion of physicians, the small number of patients analyzed, the heterogeneity of the groups of patients, and a short follow-up period. Nevertheless, the present data from more than 100 patients treated in a single center allowed us to identify factors that were associated with the prognosis in patients with MDS after allo-HCT.

In summary, the presence of cGVHD significantly reduced the risk of relapse and improved OS without increasing the incidence of NRM in patients with MDS. We also found that the presence of cGVHD significantly improved OS in high-risk patients or the RIC group, which suggests that the GVL effect may be beneficial in high-risk patients who do not receive intensive preparative regimens. For elderly or unfit patients with MDS, allo-HCT with RIC regimens was a potentially curative therapeutic option comparable with MAC regimens. As the major causes of treatment failure were disease relapse and progression, the treatment strategies to reduce the risk of relapse before and after allo-HCT are still a significant consideration for patients with high-risk MDS.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

N.H. designed the study, prepared the data file, performed the analysis, interpreted data, and wrote the manuscript; S.K was primarily responsible for the study design, data analysis, and interpretation of the data; K.O., T.K., Y.K., A.S., Y.I., R.U. and T.T. provided the patients' data; S-W.K., Y.T., and Y.H. interpreted data and reviewed the manuscript; K.T. supported the statistical analysis; T.F. provided the patients' data, interpreted data, and helped to write the manuscript.

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