Tissue damage caused by the conditioning regimen, complicated infections, and allogeneic immune reactions are the primary factors that are associated with the initial elevation of CRP early in the course of allogeneic HSCT. Consequently, it can be speculated that a reduced-intensity conditioning regimen results in decreased cytokine release and a resultant lower CRP value, which may lead to less chance of developing GVHD. Although the RIST regimens we used were relatively dose-intense, in this retrospective review we still found that CRP levels tended to be decreased after RIST compared to conventional myeloablative transplantation, particularly in a related compared to an unrelated transplantation setting. Because augmentation of allogeneic immune and inflammation reactions may induce a higher CRP value, we speculate that the benefit of RIST is diminished when a strong allogeneic reaction is induced, as in cases of unrelated transplantation.

To further evaluate the relationship between a higher CRP value during neutropenia and common risk factors associated with transplantation, we performed a multivariate analysis and showed that unrelated donor, HLA mismatch, and male sex were associated with higher CRP values. Additionally, from the finding in the multivariate analysis that unrelated donor and HLA mismatch were independently associated with high CRP, we surmised that the degree of genetic disparity might be associated with higher CRP during neutropenia. Based on a consideration of these findings together, we think that a higher CRP value may reflect the degree of tissue damage because of the transplant regimen and the subsequent magnitude of allogeneic immune reactions. Nevertheless, our analysis was hampered, because in Japan only BM and CB are allowed for unrelated transplantations, and most transplantations with a related donor use PBSC as a stem cell source. In these settings, a theoretically longer neutropenic period after unrelated BM or CB transplantation might be associated with a higher risk of infection, which could lead to higher CRP, as shown in this study.

In this study, the primary causes of death in the low-CRP group were mainly relapse and progression, whereas in the high-CRP group this was NRM. Notably, the observation that the relapse rate was higher in the low-CRP group than in the high-CRP group, as previously suggested by Min et al. [23], may further support our hypothesis that serum CRP values represent overall inflammation and cytokine production, which paves the way to GVHD and related graft-versus-leukemia (GVL) effects. A possible reason for this finding is that a low CRP level resulted in a lower incidence of GVHD and a resultant decrease in the GVL effect, or the high-CRP group developed earlier and more-frequent death from NRM compared to the low-CRP group, which left fewer patients for evaluation of the later occurrence of relapse.

In conclusion, our results suggest that the CRP value in the neutropenic period before engraftment in patients undergoing allogeneic HSCT may be a net surrogate marker of early inflammation that leads to the development of aGVHD/cGVHD and subsequent NRM, as has been proposed in mouse models. The intensity of the conditioning regimen, infectious diseases, and degree of allogeneic immune response attributed to HLA compatibility and the stem cell source may be the major factors that predict higher CRP values. Based on the results of this retrospective study, future clinical studies to evaluate the feasibility of earlier intervention and adjustment of the procedure for preventing GVHD and NRM based on monitoring of the early CRP value are warranted.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Functional analysis of cytomegalovirus-specific T lymphocytes compared to tetramer assay in patients undergoing hematopoietic stem cell transplantation

Y Morita-Hoshi[1,2], Y Heike[1], M Kawakami[1], T Sugita[3], O Miura[2], S-W Kim[1], S-I Mori[1], T Fukuda[1], R Tanosaki[1], K Tobinai[1] and Y Takaue[1]

[1]*Department of Medical Oncology, National Cancer Center Hospital, Tokyo, Japan;* [2]*Department of Hematology and Oncology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan and* [3]*Cellular Immunology Section, SRL Inc., Hachioji-city, Tokyo, Japan*

In order to evaluate whether we could predict reactivation of CMV by monitoring the number of CMV-specific cytotoxic T-lymphocytes (CTL), tetramer analysis was performed in 37 patients who underwent hematopoietic stem cell transplantation (HSCT). The results disclosed that the mean number of CMV-specific CTL at day 30 did not differ among patients who developed CMV antigenemia (22/μl) and those who did not (12/μl). Serial tetramer analysis showed that 21% of the patients had >10/μl CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than 10/μl CMV-specific CTL at the onset of CMV disease. Intracellular staining upon stimulation by CMV lysates and peptide in patients with CMV colitis revealed that both IFN-γ producing CD4+ and CD8+ lymphocytes were suppressed at the onset of CMV colitis (1.6 and 8/μl), which increased with recovery of the disease (19 and 47/μl). These data suggest that it is difficult to predict CMV reactivation solely by the number of CMV-specific CTL. We suggest that additional functional analysis by intracellular cytokine assay may be useful for immunomonitoring against CMV.

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Introduction

Reactivation of CMV is one of the major complications in patients undergoing hematopoietic stem cell transplantation (HSCT) and is significantly related to morbidity and mortality despite the recent development of potent antiviral medications.[1,2] The decision to administer antiviral therapy is currently based on the clinical risk and the detection of viremia by various methods including PCR for CMV-derived DNA or CMV antigenemia assay. However, treatment with antiviral drugs such as ganciclovir and foscarnet increases the risk for secondary graft failure and other infectious complications due to myelotoxicity. To optimize the therapy with minimum drug exposure, it is important to monitor the recovery of CMV-specific immunity accurately. For this purpose, tetramer-based monitoring of CMV-specific cytotoxic T-cells (CTL) has been widely performed in patients with an HLA-A02 or HLA-B07 serotype.[3–11] Some of the results have demonstrated that the reconstitution of CMV-specific CTL as evaluated by quantitative tetramer to levels >10–20/μl is adequate for protection against CMV infection.[5–7] However, some patients with CMV-specific CTL above this level still experience CMV reactivation.[9] It has also been reported that the cellular response to CMV in immunosuppressed patients reflects functional impairment,[10] and CMV reactivation following HSCT has been shown to be associated with the presence of dysfunctional CMV-specific T-cells.[11] Therefore, by itself, the quantification of CMV-specific CTL seems to be insufficient and a simultaneous qualitative analysis of CMV-specific lymphocytes is needed. Furthermore, it is essential that we should develop a universal monitoring method, which is not limited to HLA to cover larger populations, since an epitope that is potent enough for immunomonitoring is not obtained in some HLA types such as HLA-A24.[12] In this study, simultaneous functional analysis of CMV-specific lymphocytes by intracellular cytokine assay upon stimulation with CMV lysate and antigen peptide were performed with tetramer-based CTL quantification in patients who underwent HSCT to identify an optimal monitoring system.

Materials and methods

Study patients

CMV seropositive patients with an HLA-A*0201 or HLA-A*0206 genotype who had undergone allogeneic non-T-cell

Correspondence: Dr Y Takaue, Director, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: ytakaue@ncc.go.jp
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depleted-HSCT between February 2002 and May 2005 were included in this study. Patients were eligible with the availability for 160 days of follow-up. The study was approved by the Ethics Committee and a written informed consent was given by all patients. Peripheral blood samples were obtained at days 30 ± 7 and 60 ± 7 after transplantation. When patients agreed to additional sampling, additional samples were obtained every 2–3 weeks. The median age of studied patients was 52 (21–68). The genotype for HLA-A02 in 37 eligible patients was HLA-A*0201 in 20 patients, HLA-A*0206 in 16 patients and both the HLA-A*0201 and HLA-A*0206 genotypes in one patient. Nine patients received BMT from an unrelated donor, two received BMT from a related donor and the remaining 26 received peripheral blood HSCT from a related donor. With regard to the conditioning regimen, 11 patients received a conventional regimen that included 120 mg/kg CY plus 16 mg/kg BU or 120 mg/kg CY plus 12 Gy of TBI, whereas 26 received a reduced-intensity regimen with 0.66 mg/kg cladribine (2-chlorodeoxyadenosine) plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU. For patients who received a graft from an unrelated donor or DNA-mismatched donor, 4 Gy of TBI or 5 mg/kg of rabbit antithymocyte globulin (ATG) were added to reduced-intensity conditioning.

Diagnostic tests for CMV infection and CMV disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. All patients and 31 donors (84%) were seropositive for CMV. CMV antigenemia was monitored weekly after engraftment to day 60, and at longer intervals thereafter, by using the immunocytochemical detection of pp65 antigen in leukocytes. Test results were considered to be positive when more than one cell per 50 000 leukocytes was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ. Pre-emptive antiviral therapy was given with an antigenemia of more than 10 positive cells per 50 000 leukocytes, which we defined as high antigenemia. The initial therapy was ganciclovir 5 mg/kg once per day, which was adjusted according to the follow-up CMV antigenemia value.

Peptide and CMV antigen

A >80% pure HLA-A02-binding peptide NLVPMVATV (AA 495–503, referred to as NLV peptide) from the CMV pp65 phosphoprotein was obtained using high-performance liquid chromatography (Qiagen, Tokyo, Japan).

Tetramer staining

Tetramer staining was performed as recently described.[13] Briefly, 5 µl CD8-FITC, CD4-PC5, CD19-PC5, CD13-PC5 and 2 µl PE-conjugated tetrameric HLA-A*0201 NLV peptide complex (CMV-tetramer), purchased from Beckman Coulter Inc. (Fullerton, CA, USA), were added to 100 µl heparinized blood and incubated for 30 min. After RBC were lysed and washed twice, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). More than 20 000 cells in the lymphocyte gate were acquired and analyzed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ CMV-tetramer-positive fraction of the lymphocyte gate was defined as CMV-specific CTL.

Intracellular cytokine assay

Intracellular cytokine staining was performed as recently described[14] with the following modifications. Peripheral whole blood (1 ml) was stimulated for 6 h at 37 °C with 10 µg/ml NLV peptide or 1 µg/ml CMV lysate (Advanced Biotechnologies, Colombia, MD, USA), in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson, 1 µg/ml each). Breferdin A (Sigma, St Louis, MO, USA; 10 µg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 µg/ml staphylococcal enterotoxin B or phosphate-buffered saline. Samples were lysed, permeabilized and stained with 2.5 µl CD69-FITC, 20 µl IFN-γ-PE, 0.6 µl CD3-APC and 10 µl CD8− or CD4− PerCP. More than 10 000 cells in the lymphocyte gate were acquired and analyzed using an FACS Calibur. The cells were gated on the CD3+ fraction of the lymphocyte gate and the proportion of IFN-γ and CD8 or CD4 was analyzed. CD69 was used as a marker for activated T-cells.

Statistical analysis

The difference between groups was compared with the Wilcoxon–Mann–Whitney *U*-test and the probabilities of $P<0.05$ were defined as statistically significant.

Results

Tetramer staining

CMV antigenemia was observed in 27 patients (73%) between day 23 and day 56 (median, day 34) after transplantation; 13 (35%) of them had a peak antigenemia level of >10/50 000 leukocytes (high antigenemia) which required ganciclovir therapy and four (11%) subsequently developed CMV disease. The median number of leukocytes and lymphocytes were 3500 (1300–17 200)/µl and 576 (228–3333)/µl at day 30 and 3900 (1400–9700)/µl and 1018 (192–6790)/µl at day 60, respectively. The median percentages of CD4+ and CD8+/lymphocytes were 35% (7–64%) and 38% (20–83%) at day 30 and 25% (6–37%) and 52% (27–83%) at day 60, respectively.

The tetramer analysis showed that the mean and median number of CMV-specific CTL at day 30 was, respectively, 11 and 1.9/µl for patients without CMV antigenemia, 23 and 7.8/µl for those with antigenemia, 33 and 15/µl for those with peak antigenemia <10/50 000, 12 and 3.7/µl for those with high antigenemia, and 21 and 2.4/µl for those who developed CMV disease. There was no significant correlation between the number of CMV-specific CTL and the incidence or severity of CMV antigenemia ($P>0.05$) (Figure 1).

To further evaluate the accurate number of CMV-specific CTL at the onset of CMV antigenemia, serial analysis of CMV-specific CTL was performed weekly in 14 patients (Figures 2 and 3). Patient's characteristics are shown in Table 1. CMV antigenemia was observed in 12 patients, and five of them (UPN1-5) developed high

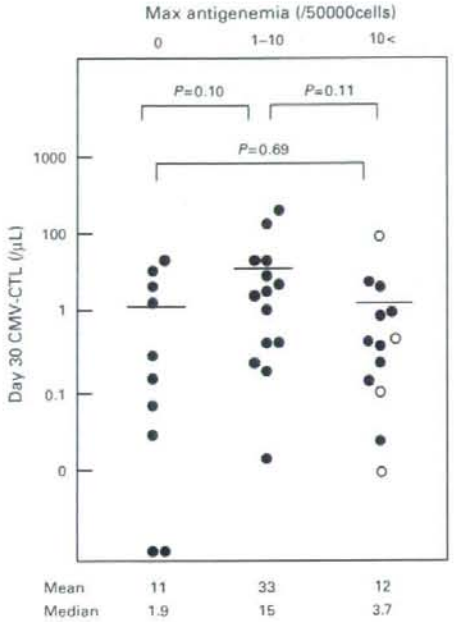

Figure 1 The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50000, who had antigenemia of >10/50000. The outlined circle ○ indicates patients who developed CMV colitis.

antigenemia, including three (UPN1-3) with CMV colitis. The mean and median number of CMV-specific CTL at the first detection of CMV antigenemia was 21/μl and 4.7 (0–100)/μl in the 12 patients, and three (UPN2, 13, 14) showed >10/μl. For those who did not require antiviral therapy (UPN6-14), the number of CMV-specific CTL was widely ranged. While UPN6-8 showed <10/μl throughout the observation time, the maximum CTL count was >200/μl for UPN12-14. The number of CMV-specific CTL for UPN1 and UPN2 who developed CMV colitis showed >10/μl, which was 14 and 80/μl when diarrhea occurred, and 88 and 63/μl, respectively at the time of colon biopsy which proved CMV colitis.

It has been demonstrated that in patients coexpressing HLA-A02 and HLA-B07, CMV-specific cellular immune responses restricted by HLA-B07 dominate those restricted by HLA-A02, possibly because CD8+ T cells specific for dominant epitopes are able to suppress immune responses to less favored epitopes.[8] The allele frequency of HLA-B07 is low (5.2%) among Japanese[15] and only one patient coexpressed HLA-B07 in this study. We did not exclude this patient (UPN14) from the analysis because the number of HLA-A02-restricted CMV-specific CTL in this patient was 9.5/μl on day 30 and the maximum value reached 243/μl on day 128 suggesting that the coexpression of HLA-B07 seems not to have affected the immunoresponse of HLA-A2 in this patient.

Intracellular cytokine assay

Upon stimulation with CMV lysate, intracellular IFN-γ staining among five patients (UPN1–5) who developed high

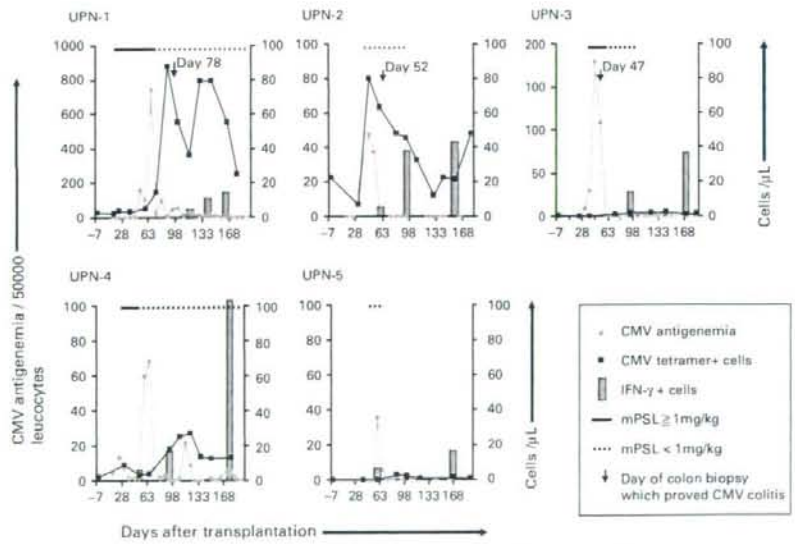

Figure 2 Serial analysis of patients who had high antigenemia of >10/50000. ■ indicates CMV-specific CTL as evaluated by tetramer assay, ♦ indicates CMV antigenemia, gray bar indicates the number of IFN-γ + cells/μl peripheral blood when stimulated with CMV lysate, the solid line indicates methylprednisolone administration of 1 mg/kg/day or more, the dashed line indicates corticosteroid administration less than 1 mg/kg/day and ↓ indicates the day of colon biopsy which CMV disease was diagnosed. UPN1, 2, 3 developed CMV disease. Intracellular IFN-γ was undetectable on day 60 and day 90 for UPN1 and on day 60 for UPN3.

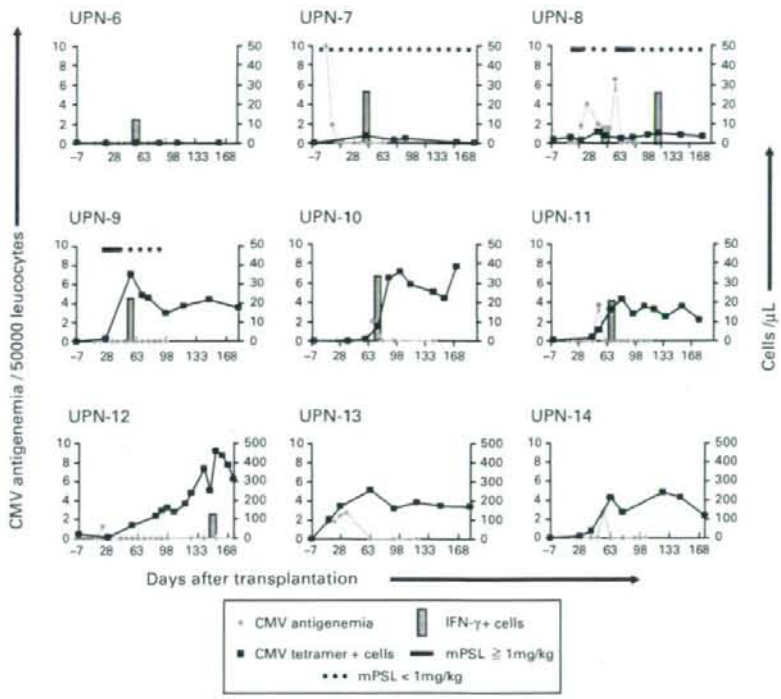

Figure 3 Serial analysis in patients with CMV antigenemia of <10/50000 or patients without CMV antigenemia. The legends are the same as Figure 2. Intracellular cytokine was not assessed for UPN13 and UPN14.

Table 1 Patients' characteristics

ID	*Age*	*HLA-A locus*	*Primary disease*	*Conditioning regimen*	*GVHD prophylaxis*	*Stem cell source*	*CMV serology*		*Max CMV-Ag*	*CMV disease*
							Recipient	*Donor*		
UPN-01	63	0201, 0206	CML (AP)	CdA/BU	CSP→TAC	PB	+	+	740	+
UPN-02	57	0201	NHL (DLBCL)	CdA/BU	CSP	PB	+	+	48	+
UPN-03	49	0201	NHL (low grade)	CdA/BU	CSP→TAC	PB	+	+	178	+
UPN-04	54	0206	MCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	68	–
UPN-05	59	0206	AML	CdA/BU/TBI	CSP+sMTX	UBM	+	+	35	–
UPN-06	66	0206	MDS (RA)	Flu/BU	CSP+sMTX	PB	+	–	0	–
UPN-07	61	0201	NHL (low grade)	Flu/BU/ATG	CSP	UBM	+	+	10	–
UPN-08	62	0201	AML	CdA/BU	TAC	PB	+	+	6.5	–
UPN-09	43	0201	MDS (RA)	BU/CY	CSP+sMTX	UBM	+	–	0	–
UPN-10	41	0206	AML	BU/CY	CSP+sMTX	RBM	+	+	2.1	–
UPN-11	54	0201	NHL (low grade)	Flu/BU	CSP+sMTX	PB	+	+	3.7	–
UPN-12	32	0206	RCC	CdA/BU	CSP	PB	+	+	2.8	–
UPN-13	42	0206	PCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	2.8	–
UPN-14	43	0206	RCC	CdA/BU/ ATG	CSP	PB	+	+	1.3	–

Abbreviations: ATG = antithymocyte globulin; CdA = cladribine; CML (AP) = CML (accelerated phase); CSP = cyclosporine; DLBCL = diffuse large B-cell lymphoma; Flu = fludarabine; MCL = mantle cell lymphoma; MDS (RA) = myelodysplastic syndrome (refractory anemia); NHL = non-Hodgkin lymphoma; PB = peripheral blood; PCL = plasma cell leukemia; RBM = related bone marrow; RCC = renal cell carcinoma; sMTX = short term methotrexate; TAC = tacrolimus; UBM = unrelated bone marrow.

antigenemia and required antiviral therapy showed that the mean number of IFN-γ-producing cells was 3.6 (0–6.7)/μl at day 60, which subsequently increased to 72 (15–250)/μl at day 160. As for three patients with CMV colitis (UPN1–3), only one patient (UPN2) had detectable level of IFN-γ-producing cells (4.8/μl) at the time of disease

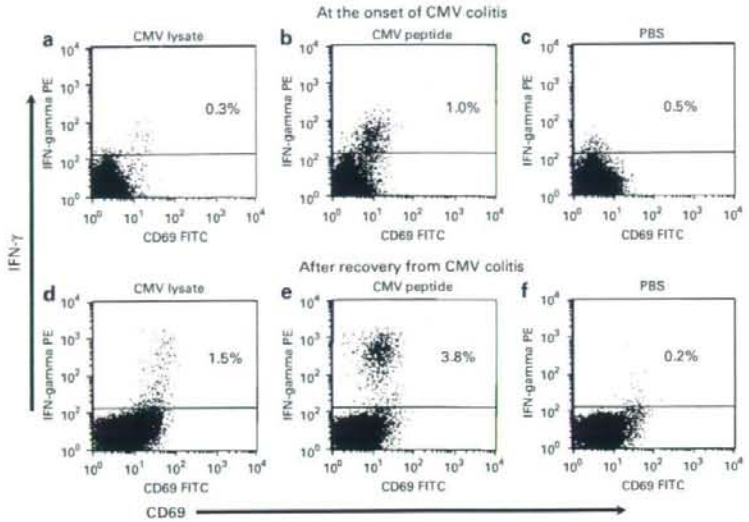

Figure 4 Intracellular cytokine assay in a patient with CMV colitis (UPN2). The samples were taken at the onset of CMV colitis (**a**–**c**) and after recovery from CMV colitis (**d**–**f**). The numbers of IFN-γ-producing cells on lysate stimulation (**a**, **d**) and peptide stimulation (**b**, **e**) both increased after recovery from CMV colitis. (**c**) and (**f**) are negative controls.

onset and were undetectable for the other two patients, which remained negative until day 90 for UPN1. The mean number of IFN-γ+ cells subsequently increased to 19 (5–38)/μl after recovery from CMV disease (Figures 2, 4a and d). Among the patients who did not require antiviral therapy, the IFN-γ-producing cells were all >10/μl at day 60.

When stimulated with CMV peptide, IFN-γ-producing cells numbered 8 (0–16)/μl at the time of disease onset with a subsequent increase to 47 (15–95)/μl after recovery from CMV disease (Figures 4b and e).

Regarding the phenotype of IFN-γ-producing cells, median of 81% (76–100) were CD4+ and <20% were CD8+ upon stimulation by CMV lysate. The staining of IFN-γ was brighter in CD4+ than in CD8+ cells and CD69 was positive for both CD4+ and CD8+ fraction. IFN-γ-producing cells were CD69 low positive and median of 42% (25–68) were CD8+, while the rest were CD8−/CD4− phenotype upon CMV peptide stimulation.

Discussion

Our results showed that it is difficult to predict CMV infection by the number of CMV-specific CTL alone as this did not correlate with the incidence and severity of CMV infection. While UPN1 and UPN2 developed CMV colitis after the recovery of sufficient number of CTL, UPN6, UPN7 and UPN8 did not require antiviral therapy despite low CMV-specific CTL. These results showed that CMV disease could occur after HSCT even in patients with >10/μl CMV-specific CTL as evaluated by tetramer assay, which has been considered to be sufficient to protect against CMV infection.[5,7]

CMV-specific CTL emerged immediately following the detection of antigenemia in most patients, suggesting that CMV infection can be a trigger for the recovery of CMV-specific immunity. However, UPN9 had recovery of CMV-specific CTL at day 60 even though his CMV antigenemia and CMV DNA as evaluated by PCR were negative throughout the course.

On the other hand, intracellular analysis revealed that IFN-γ production in both CD4+ and CD8+ T lymphocytes was depressed in patients with high antigenemia or CMV disease and this had subsequently recovered at disease resolution. Functional analysis methods for CMV-specific immune response by flow cytometry have been established,[16] and it was reported that patients who developed CMV disease after SCT had no detectable IFN-γ production by CD3+/4+ T-cells upon CMV AD-169 antigen stimulation.[17] It has also been demonstrated that levels of IFN-γ-producing CD4+ cells less than one cell/μl and CD8+ less than three cells/μl upon stimulation by CMV-infected autologous dendritic cells are not protective against recurrent infection.[18] As assessed by IFN-γ ELISPOT assay, the threshold level for protection against CMV reactivation was estimated as over one cell/μl peripheral blood upon CMV pp65 peptide stimulation.[19] The number of IFN-γ-producing cells upon CMV lysate stimulation were above ten cells/μl among patients whose antigenemia was <10/50 000 cells in our study, which may be sufficient for protection against CMV reactivation. It is difficult to determine the exact threshold level for protection against CMV since IFN-γ production differs among various stimulating agents. Also the magnitude of response is higher in the cytokine flow cytometry assay while the cytokine flow cytometry assay was less likely than the ELISPOT assay to detect low-level responses.[20]

Several studies on HIV-infected patients have shown the availability of analyzing the phenotype and other cytokine production of virus-specific T-cells such as IL-2, TNF-α.[21,23] It has been demonstrated that virus-specific T-cells, which produce both IFN-γ and IL-2 are important in virus-specific immunity, and that IFN-γ/IL-2 secreting CD8+ T-cells were CD45RA−/CCR7− phenotype and correlated with that of proliferating T-cells, whereas single IFN-γ-secreting cells were either CD45RA−/CCR7− or CD45RA+/CCR7−.[22] Another study has shown that immunorestored patients had increased levels of circulating CMV-specific CD8+ T-cells with 'early' (CD27+/CD28+/CD45RA+, CD27+/CD28+/CD45RA−) and 'intermediate' (CD27−/CD28+/CD45RA−) phenotype.[23] Only IFN-γ production was assessed in our study, however higher-order flow cytometry might have added more discriminatory value. Foster *et al*,[24] demonstrated that CMV-specific CD4+ T-helper cells show the same reconstitution kinetics as CD8+ CTL. Thus, functional analysis of lymphocytes upon lysate stimulation that can be used to assess both CD4+ and CD8+ cells is a useful tool for monitoring T cell immunity against CMV in patients after HSCT. This method is more widely applicable than peptide stimulation or tetramer assay, since it is not restricted to HLA or a single epitope. However, peptide stimulation and tetramer assay may still be a major procedure in the analysis of CD8+ T-cells, since tetramers are widely applied to adoptive immunotherapy of CMV[25] and the dominant population of IFN-γ-producing cells upon lysate stimulation was CD4+. Previous study has demonstrated that flow cytometry following stimulation of PBMC with pp65 and immediate early (IE)-1 peptide pools consisted of 15-aa peptides was highly sensitive and specific in predicting the presence of recognized epitope in the respective proteins.[26] Furthermore, it has been shown that IE-1-specific responses were more important in protective immunity than pp65-specific responses in heart and lung transplant recipients.[27] The stimulation with comprehensive peptide pools might have better assessed both functional CD4+ and CD8+ T-cell responses. Further study is needed to identify whether IE-1 is more important than pp65 in allogeneic HSCT patients, and the significance of IE-1 in Japanese population with low allele frequency of HLA-A1 (1.8%), -B7 (5.2%) or -B8 (<1%),[15] which is known to present IE-1 epitopes.

It is likely that the patients who did not have CMV reactivation despite low CMV-specific CTL had sufficient T-cell immune-recovery against CMV since the number of intracellular IFN-γ positive cells upon CMV lysate stimulation was as high as that in patients who had recovered from CMV reactivation. As for CD8+ T cells in these patients, CTL against other CMV-epitopes besides NLV might have helped to protect against CMV. It is reported that the recovery of CMV specific T-cells is earlier in patients who received reduced-intensity conditioning compared to conventional regimen and this was delayed by the use of ATG.[19,28] Additionally, the graft source and CD3+ T-cell dose significantly influence the recovery of CMV-specific immunity.[28] The difference of immune recovery according to the conditioning regimen and graft source was not demonstrated in this study, probably due to heterogeneous patients and small sample size. Functional depression of the lymphocytes due to corticosteroid for GVHD seems to be the major cause of CMV infection as documented in all patients with high antigenemia. Moreover, 75% of the patients with CMV disease were receiving more than 1 mg/kg/day of methylprednisolone (mPSL), while among those who did not require antiviral therapy, only 13% had received 1 mg/kg/day or more mPSL. The influence of corticosteroid on the number of CMV-specific CTL is controversial. Some studies have reported that a significant reduction of CMV-specific CTL occurred with corticosteroid therapy.[6,8] Others have shown that the frequency and the absolute number of CMV-specific CD8+ T cells were similar in patients receiving corticosteroids and those who didn't, while the CMV-specific CD8+ T cells showed decreased cytokine production.[10,11] Our result was consistent with the latter observation that while the number of CMV-specific CTL does not decrease significantly with corticosteroid therapy, IFN-γ production of CMV-specific CTL is severely suppressed. Therefore, concomitant assessment of T-cell function is essential in patients after HSCT, especially in those who are receiving corticosteroid therapy.

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ORIGINAL ARTICLE

Intensive glucose control after allogeneic hematopoietic stem cell transplantation: a retrospective matched-cohort study

S Fuji[1], S-W Kim[1], S Mori[1], S Kamiya[2], K Yoshimura[3], H Yokoyama[1], S Kurosawa[1], B Saito[1], T Takahashi[1], S Kuwahara[2], Y Heike[1], R Tanosaki[1], Y Takaue[1] and T Fukuda[1]

[1]*Department of Hematology and Stem Cell Transplantation, Tokyo, Japan;* [2]*Division of Nutritional Management, National Cancer Center Hospital, Tokyo, Japan and* [3]*Biostatistics and Epidemiology Section, Center for Cancer Control and Information Services, National Cancer Center Hospital, Tokyo, Japan*

Some studies have shown that intensive glucose control (IGC) improves outcome in the intensive care unit setting. However, it is the benefit of IGC in hematopoietic SCT (HSCT) that is not well defined. Between June 2006 and May 2007, IGC was maintained prospectively after allogeneic HSCT and clinical outcomes were compared with a cohort matched for conditioning regimen, source of stem cells, age and relation to donor. A stratified Cox regression model was used. There were no significant differences in baseline clinical characteristics. The median age was 43.5 years in both groups. The primary diagnosis was a hematologic malignancy. Patients in the IGC group had a lower glucose level (least-square mean, 116.4 vs 146.8 mg per 100 ml, $P<0.001$) compared to the standard glucose control group. The incidences of documented infections and bacteremia were significantly lower in the IGC group (14 vs 46%, $P=0.004$, 9 vs 39%, $P=0.002$, respectively). IGC tended to reduce the incidence of renal dysfunction (19 vs 37%, $P=0.36$) and the elevation of C-reactive protein (18 vs 38%, $P=0.13$). This study suggests that IGC has may have a beneficial effect after HSCT. IGC should be evaluated further in a large prospective, randomized study.

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Keywords: intensive glucose control; allogeneic transplantation; hyperglycemia; C-reactive protein

Introduction

Previous studies showed that intensive glucose control (IGC), in which the target blood glucose level was set within 80–110 mg per 100 ml, reduced infections, dysfunction of organs including the liver and kidney and mortality compared to patients who received standard glucose control.[1–3] Although these results have been confirmed in several subsequent studies,[4–7] the precise mechanism that underlies this association is unclear. In animal models, it has been shown that insulin itself has a direct inhibitory effect on the inflammation process.[8,9] However in human studies, it has been suggested that these benefits could be directly attributed to IGC rather than to any pharmacological activity of administered insulin *per se*.[3,4]

Recipients of allogeneic hematopoietic SCT (HSCT), which is the most drastic therapeutic modality in patients with hematological malignancies, often suffer from serious complications including infectious diseases, GVHD and multiple organ failure. They are also at higher risk of hyperglycemia because of the use of steroids for the treatment of GVHD, the use of total parenteral nutrition (TPN), immunosuppressive drugs and infectious complications,[10,11] which makes them further susceptible to numerous serious complications including infectious diseases and multiple organ failure.[12–14] Our group previously reported that hyperglycemia during neutropenia was associated with an increased risk of acute GVHD and nonrelapse mortality (NRM) after myeloablative allogeneic HSCT,[15] and that hyperglycemia during neutropenia was associated with a higher incidence of subsequent acute GVHD. It is well known that an increase in the levels of circulating cytokines may aggravate hyperglycemia, and hyperglycemia itself could increase the levels of cytokines. This vicious cycle could lead to elevated cytokine levels, which could lead to subsequent acute GVHD. With this background, it can be hypothesized that IGC would reduce the incidence of infectious diseases, acute GVHD and organ dysfunctions after allogeneic HSCT. Therefore, we prospectively investigated the effect of IGC after allogeneic HSCT, and compared the clinical outcomes to those in a matched cohort to address whether IGC following allogeneic HSCT could improve the clinical course of patients, that is, reduction of infectious diseases and organ dysfunction, as has been shown in the intensive care unit (ICU) setting.

Correspondence: Dr Y Takaue, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan.
E-mail: ytakaue@ncc.go.jp
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Patients and methods

Patients

From June 2006 to May 2007, a total of 73 patients received allogeneic HSCT at the National Cancer Center Hospital (Tokyo, Japan); 60 patients were eligible for participation in this trial. Finally, 22 patients (36.7%) were enrolled in this IGC study to keep the blood glucose level at 80–110 mg per 100 ml, as shown in Figure 1.

Study center and organization

The National Cancer Center Hospital in Tokyo holds 600 beds. The transplant team consists of 4 full-time physicians and 26 nursing staff who oversee 26 beds in the HSCT, and the entire ward is covered by high-efficiency particulate air-filters. We regularly perform 90–120 transplants per year: 80% allogeneic and 20% autologous.

Study design

This was a case–control study to investigate the clinical benefits of comprehensive nutritional support including IGC and parenteral nutrition (PN) management, which was approved by the Institutional Review Board. A matching control group was selected among patients who received HSCT from January 2002 to March 2007 (ratio of 1:2 compared to the study group) according to the following criteria: (1) conditioning regimen (conventional myeloablative or reduced intensity), (2) source of stem cells (BM, peripheral blood or cord blood), (3) age and (4) source of donor (related or unrelated). Criteria (1–4) were essential for inclusion. As a result, 42 matched controls were selected, and a total of 64 patients were subjected to further analysis (Table 1).

Exclusion criteria

Exclusion criteria were as follows: (1) patients who received a reduced-intensity conditioning regimen for an HLA-matched related donor, as we applied GVHD prophylaxis without short-term MTX in this setting, and they had much less need for TPN and less need for intense glucose control,[16] (2) those with a poor performance status (Eastern Cooperative Oncology Group) ≥ 2, (3) those with uncontrolled infectious diseases at the beginning of the conditioning regimen and (4) those with preexisting neutropenia. We previously reported that the incidence of severe stomatitis (Common Terminology Criteria for Adverse Events (CTCAE) grade (3) was 0% after reduced-intensity SCT (RIST) from a related HLA-matched donor.[16] In this situation, the need for TPN and the incidence of hyperglycemia were quite low, compared to RIST from an unrelated donor, which included additional low-dose TBI or antithymocyte globulin (ATG) and short-term MTX or conventional SCT with a myeloablative regimen. Hence, we only included patients who received a RIST regimen from an unrelated donor, who had a higher probability of glucose-control intervention, to evaluate the beneficial effects of IGC.

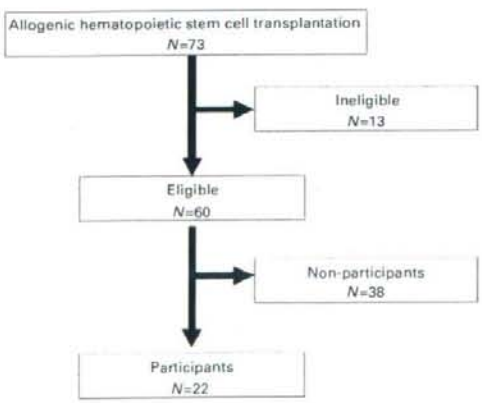

Figure 1 Trial profile.

Table 1 Patients' characteristics

Variable	N *(%)/median (range)*		P-*value*
	Intensive glucose control (n = *22*)	*Standard glucose control* (n = *42*)	
Age (years)	43.5 (17–64)	43.5 (20–66)	
<40	8 (36)	18 (43)	0.62
≥40	14 (64)	24 (57)	
Sex			
Male	9 (41)	22 (52)	0.38
Female	13 (59)	20 (48)	
Disease risk[a]			
Standard	6 (27)	16 (38)	0.39
High	16 (73)	26 (62)	
Conditioning			
CST	14 (64)	27 (64)	
BU/CY	9 (40)	18 (43)	
CY/TBI (12 Gy)	4 (18)	6 (14)	
Other	1 (5)	3 (7)	
RIST	8 (36)	15 (36)	0.96
2CdA/BU	1 (5)	1 (2)	
Flu/BU	7 (32)	14(33)	
Low-dose TBI (2–4 Gy)	3 (14)	7 (17)	
Low-dose ATG	5 (23)	10 (24)	0.92
GVHD prophylaxis			
Cyclosporin-based	7 (32)	27 (64)	
Tacrolimus-based	15 (68)	15 (36)	0.01
Short-term MTX (+)	22 (100)	40 (95)	0.30
Relation to donor			
Related	6 (27)	12 (29)	
Unrelated	16 (73)	30 (71)	0.91
Stem cell source			
Bone marrow	15 (68)	30 (71)	
PBSC	5 (23)	10 (24)	
Cord blood	2 (9)	2 (5)	0.19
HLA match			
Match	11 (50)	28 (67)	
Mismatch	11 (50)	14 (33)	0.19

Abbreviations: ATG = antithymocyte globulin; 2CdA = cladribine; CST = conventional stem cell transplantation; Flu = fludarabine; RIST = reduced-intensity stem cell transplantation.
[a]Standard-risk patients included those with acute leukemia in first complete remission, chronic leukemia in first chronic phase, MDS in refractory anemia and NHL in complete remission, and the remaining patients were categorized as high risk.

Transplantation procedures

Forty-one patients received a myeloablative conditioning regimen that included BU (orally 4 mg/kg per day × 4 days or i.v. 3.2 mg/kg per day × 4 days) plus CY (60 mg/kg per day × 2 days, $n=27$), CY plus 12 Gy TBI ($n=10$) or other ($n=4$). Twenty-three patients received a reduced-intensity conditioning regimen that included fludarabine (30 mg/m^2 per day × 6 days) or cladribine (0.11 mg/kg per day × 6 days) plus BU (oral 4 mg/kg per day × 2 days or i.v. 3.2 mg/kg per day × 2 days). Low-dose TBI (2 or 4 Gy, $n=10$) and/or low-dose ATG (total dose 5–10 mg/kg ATG-F or 5 mg/kg thymoglobulin, $n=15$) were added. GVHD prophylaxis included CYA- ($n=13$) and tacrolimus-based regimens ($n=51$), with an additional short course of MTX. G-CSF was administered in all patients from day +6 after transplantation until engraftment. Most patients received ciprofloxacin (200 mg orally three times daily) for bacterial prophylaxis after the beginning of the conditioning regimen until neutrophil engraftment. Fluconazole (100 mg once daily) was administered for fungal prophylaxis after the beginning of the conditioning regimen. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and VZV after the beginning of the conditioning regimen until immunosuppressive agents were discontinued. Prophylaxis against *Pneumocystis jiroveci* infection consisted of trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the cessation of immunosuppressive agents. Patients who developed fever during the neutropenic period were treated with cefepime or other cephalosporin, and additional agents including vancomycin, aminoglycosides and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the ANC exceeded 0.5×10^9 per l.

Glucose management protocol

In the IGC group, the blood glucose level was routinely tested every morning to adjust the dose of insulin so as to keep the level within the range of 80–110 mg per 100 ml. Owing to the presence of fewer nursing staff in the HSCT unit than in the ICU, we replaced the continuous infusion of insulin with the addition of Humulin R to the bottle of PN to control the glucose level within the target range. In TPN, we universally added at least 1 unit of Humulin R per 10 g glucose. In patients who had an elevated blood glucose level, we also added Humulin R to the bottle of PN. We monitored the glucose level at least once a day in the morning as long as the level remained within the target range of 80–110 mg per 100 ml. When the glucose level became elevated, we increased the frequency of monitoring up to 2–4 times daily. In most patients, we adjusted the dose of insulin added to the bottle of PN as described in Table 2. When the blood glucose level was >180 mg per 100 ml or the dose of insulin was high, we manually adjusted the dose of Humulin R and administered insulin subcutaneously according to the attending physician's discretion. S.c. insulin administration usually consisted of 3–5 units at the beginning, and, if this was insufficient, the dose was manually adjusted by 2–4 units. When the patients received high-dose systemic steroid such as methylprednisolone 1–2 mg/kg per day for GVHD, we used the preprandial s.c. injection of insulin Aspart (NovoRapid) three times daily to avoid postprandial hyperglycemia and adjusted the dose according to the amount of food intake and the postprandial glucose level. When patients exhibited nausea, anorexia or vomiting, the amount of food intake became unstable. In such situations, insulin Aspart was injected immediately after the meal. When food intake was <50%, the dose was reduced or discontinued. Routine glucose monitoring was continued until PN was stopped, whereas the blood glucose level was maintained within the target range. Daily caloric intake was calculated by the dietitians. We tried to maintain oral intake as much as possible by using a suitable diet in jelly or liquid form. A dietitian adjusted the dose of supplemental PN to maintain the total caloric intake over 1.0 × basal energy expenditure (BEE), and if the glucose level was stable, the nutritional intake could be increased up to 1.5 × BEE. The glucose concentration in PN was usually started at 7.5% glucose as supplemental PN. The concentration was gradually increased to 12%, and, if necessary, this was further increased up to 18% to meet the target caloric intake. A lipid emulsion was also used to supply 10–30% of total caloric intake. The minimal total nutritional intake was set at 1.0 × BEE because a retrospective analysis at our institute showed that caloric intake of more than 1.0 × BEE was not associated with clinically significant wt loss.[17] To improve the glucose control, this level was set to be slightly lower

Table 2 Protocol for adjustment of Humulin R

Glucose level (mg per 100 ml)	Adjustment of Humulin R
BS ≤ 40	i.v. 50% glucose 20 ml and recheck the glucose level Reduce the dose of Humulin R to 40–60% of the original dose
40 ≤ BS < 60	i.v. 50% glucose 20 ml and recheck the glucose level Reduce the dose of Humulin R to 60–80% of the original dose
60 ≤ BS < 80	i.v. 50% glucose 20 ml and recheck the glucose level Reduce the dose of Humulin R to 70–90% of the original dose
80 ≤ BS ≤ 110	No change
110 < BS < 130	Increase the dose of Humulin R to 110–120% of the original dose
130 ≤ BS < 150	Increase the dose of Humulin R to 120–130% of the original dose
150 ≤ BS < 180	Increase the dose of Humulin R to 130–150% of the original dose
BS ≥ 180	Manually adjust the dose of Humulin R combined with sliding subcutaneous insulin administration

Abbreviation: BS = blood sugars.

than the recommendation in the HSCT setting (1.3–1.5 × BEE,[18]). There are two beneficial aspects of this protocol: we could maintain the minimal caloric intake with supplemental PN and we could immediately start insulin as required after the introduction of PN. The SGC group was managed without a specific protocol for nutrition practice and glucose control, although we routinely monitored blood glucose at least three times weekly to avoid severe hyperglycemia (blood glucose >200 mg per 100 ml).

Outcome measures

Serially monitored glucose values were compared between the IGC group and the SGC group. We also analyzed the association between the mean glucose level during monitoring and the infection rate in both the SGC group and IGC group. Mean glucose levels were estimated for each patient and were categorized as follows: 80–110, 111–140, 141–179 and >180. Glycemic variability, defined as the s.d. of the mean glucose value, was also analyzed. The outcome measures were time to the occurrence of documented infectious complications within 100 days after HSCT, time to each organ dysfunction defined as described below, time to grades II–IV and grades III–IV acute GVHD and time to NRM. These were calculated from the date of the start of the conditioning regimen. Organ dysfunction was defined with reference to van den Berghe[5–7] as follows: (1) hypercreatininemia; serum creatinine level ⩾2.0 mg per 100 ml or more than twice the baseline, (2) hyperbilirubinemia; serum total bilirubin level ⩾2.0 mg per 100 ml and (3) increased inflammatory markers; serum C-reactive protein (CRP) level ⩾15 mg per 100 ml. In our institute, the CRP level was routinely monitored at least three times a week, as we previously reported that the preengraftment CRP level may predict a subsequent occurrence of acute GVHD and NRM after allogeneic HSCT.[19] These results suggested that CRP might be useful not only as a marker of infectious diseases but also as a surrogate marker for produced cytokines. Therefore, the serial changes of CRP level were compared between the two groups. Acute GVHD was graded by the consensus criteria.[20]

Statistical analyses

Baseline characteristics were summarized using descriptive statistics. The Student's t, χ^2 and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. The probability of documented infectious complications and organ dysfunction were calculated using Kaplan–Meier estimates. A stratified Cox regression model, which accounts for the matched-cohort design, was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). On the basis of 64 patients, the study has an approximately 80% power to detect a HR of 0.5 for documented infections. The glucose values, measured repeatedly, were compared between groups using a repeated-measure analysis with a linear mixed-effect model. A level of $P<0.05$ was defined as statistically significant. All P-values are two-sided. All analyses were performed using SAS version 9.1.3 (Cary, NC, USA).

Results

Patient characteristics

Table 1 lists the patients' clinical and transplantation characteristics. Patients and transplantation characteristics were well balanced with the application of matching criteria. Nevertheless, in the IGC group, more patients received tacrolimus for GVHD prophylaxis (68 vs 36%, $P=0.01$) and more had a previous transplantation (32 vs 7%, $P=0.01$). The median duration of follow-up in surviving patients was 299 days (range, 78–607 days) in the IGC group and 1146 days (range, 329–1774 days) in the SGC group.

Glycemic control

Duration of monitoring and number of tests. The median duration of glucose monitoring and intervention in the IGC group was 38 days (range, 24–70 days) after the start of the conditioning regimen. The total number of glycemic monitorings was 867 and 1094 in the SGC group and IGC group, respectively.

Mean values and distribution of values. Patients in the IGC group had a lower glucose level (least-square mean, 116.4 vs 146.8 mg per 100 ml, $P<0.001$) than the SGC group. The trend of the glucose value is shown in Figure 2a. All glycemic results for the SGC and IGC groups were stratified into six levels: <40, 40–79, 80–110, 111–140, 141–179 and ⩾180, as shown in Figure 2b.

Hypoglycemia

In the IGC group, the incidence of mild hypoglycemia (CTCAE grades 1–2, glucose level 40–69 mg per 100 ml) was significantly higher than that in the SGC group (11 vs 3 patients, $P<0.001$). Although one patient (4.5%) in the IGC group who was diagnosed as type 2 diabetes mellitus developed severe hypoglycemia (CTCAE grade 3, glucose level 30–39 mg per 100 ml) with faintness, no patient developed seizure or loss of consciousness.

Glycemic variability

The mean glycemic variability in the SGC group and IGC group was 37.2 mg per 100 ml (range, 10.1–121.7 mg per 100 ml) and 27.5 mg per 100 ml (range, 11.3–46.6 mg per 100 ml), respectively, and glycemic variability in the IGC group tended to be lower than that in the SGC group ($P=0.07$).

TPN and insulin dosing

The percentage of patients who received TPN was 60% (25 patients) and 77% (17 patients) in the SGC group and the IGC group, respectively. The mean duration of TPN was 9 days (range, 0–35) and 13 days (range, 0–38) in the SGC group and IGC group, respectively. There was a tendency for more patients in the IGC group to receive TPN compared to the SGC group, but this difference was not statistically significant. The mean maximal dose of insulin (median (range), 51 (0–100) vs 2 (0–110) IU, $P<0.001$) and the mean maximal dose of insulin per 1 g parenteral glucose

were significantly higher in the IGC group (median (range), 0.22 (0–0.71) vs 0.003 (0–0.4) IU/g glucose, $P<0.001$).

Infections

Table 3 summarizes the results. In the IGC group, dramatically fewer patients developed documented infections within 100 days compared to the SGC group, as shown in Figure 3.

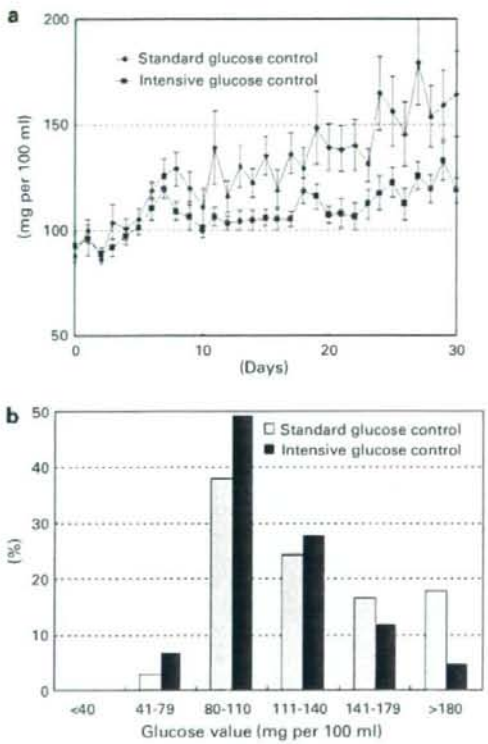

Figure 2 Serial changes in the mean glucose level in the intensive glucose control (IGC) and standard glucose control (SGC) groups. Values are mean + s.e. (**a**). The distribution of the glucose values in IGC and SGC is shown as a histogram (**b**).

Relation to mean glucose level

We also analyzed the association between the mean glucose level during monitoring and the infection rate in both the SGC and IGC groups. The incidence of infection was 34, 17, 67 and 40%, respectively, with mean glucose levels of 80–110, 111–140, 141–179 and ⩾180. When we compared a lower glucose-level group (mean glucose level of 80–140) with a higher glucose-level group (mean glucose level of >140), the incidence of infection was significantly higher in the latter group (28 vs 57%, $P=0.042$). When we assessed only patients with a lower glucose level, the IGC group tended to show a lower incidence of infectious diseases than the SGC group (14 vs 41%, $P=0.061$).

Relation to glycemic variability

We also analyzed the association between glycemic variability and the infection rate. The mean glycemic variability in patients with and without infection was 34.6 mg per 100 ml (range, 10.5–121.7 mg per 100 ml) and 33.3 mg per 100 ml (range, 10.1–110.6 mg per 100 ml), respectively, with no significant difference. As the importance of glycemic variability could vary among patients

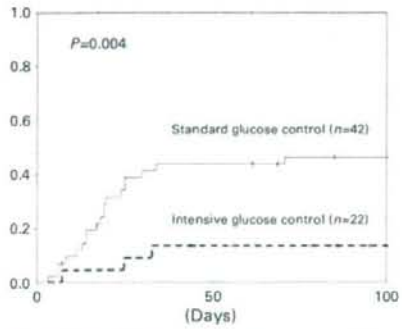

Figure 3 Probability of documented infections in the IGC and SGC groups.

Table 3 Incidence of infectious diseases and organ dysfunction

Variable	N (%)/median (range)			
	Intensive glucose control n = 22 (%)	*Standard glucose control* n = 42 (%)	*HR (95% CI)*	P-value
Documented infection	13	46	0.17 (0.04–0.75)	0.004
Bacteremia	9	39	0.10 (0.01–0.74)	0.002
Organ dysfunction				
Hypercreatininemia[a]	19	37	0.60 (0.19–1.88)	0.36
Hyperbilirubinemia[b]	28	31	1.05 (0.38–2.91)	0.93
Increased inflammatory markers[c]	18	38	0.45 (0.15–1.37)	0.13

Abbreviations: CI = confidence interval.
[a]Serum creatinine level ⩾2.0 mg per 100 ml or more than twice of baseline.
[b]Serum bilirubin level ⩾2.0 mg per 100 ml.
[c]Serum C-reactive protein level ⩾15 mg per 100 ml.

with different mean glucose levels,[21] we divided the patients into two groups based on mean glucose level 80–140 or 140+ and then determined whether glycemic variability was associated with an increased incidence of infections. However, there was no significant association between glycemic variability and the incidence of infections in both groups.

CRP levels

Figure 4 shows serial changes in the CRP level. Even though there was no difference in the CRP level between the two groups at the beginning of the conditioning regimen, the CRP level was significantly elevated in the SGC group compared to that in the IGC group 15 days after the beginning of the conditioning regimen, and this trend continued up to 40 days ($P<0.05$). The maximal CRP level during the neutropenic period in the IGC group was significantly lower than that in the SGC group (median (range), 6.9 (0.9–16.3) vs 11.5 (1.6–37.3), $P=0.007$).

Other clinical outcomes

The probability of grades II–IV acute GVHD within 100 days was 28 and 37% in the IGC and SGC groups (HR 1.05, 95% CI 0.38–2.91, $P=0.93$). The incidences of grades III–IV acute GVHD and NRM within 100 days were low in both groups (one and two patients, and one and one patient, in the IGC and SGC groups, respectively).

Discussion

This is the first study to evaluate the outcomes in allogeneic HSCT patients who were treated with a glucose management protocol. A salient finding of this study is that the incidence of documented infections, especially the incidence of bacteremia, was significantly lower in the IGC group than in the SGC group, as in a previous report in the ICU setting.[1] Moreover, there tended to be fewer organ dysfunctions in the IGC group, albeit this difference was not statistically significant. Furthermore, the CRP level, which might be a surrogate marker for produced cytokines,[19] was significantly lower in the IGC group than in the SGC group, as shown in Figure 4. Even though this study did not have enough power to detect a decrease in acute GVHD and NRM, it could be anticipated that IGC could reduce the CRP level, which would lead to a reduced incidence of acute GVHD and NRM.

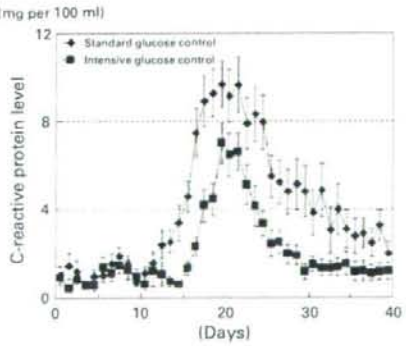

Figure 4 Serial change in the CRP level in the IGC and SGC groups. Values are mean + s.e.

This study has several limitations. One limitation is that only 64 patients were analyzed with no sufficient power to demonstrate any statistically significant changes in the incidences of organ dysfunctions, which was similar to the result in a previous report in the ICU.[1,2] An additional limitation was that the control of the glucose level could be suboptimal. This could be because of the glucose control protocol, which included monitoring of glucose level and the administration of insulin. With regard to the administration of insulin, we replaced the continuous infusion of insulin with the addition of Humulin R to the bottle of PN to control the glucose level within the target range because of the presence of fewer nursing staff in the HSCT unit than in the ICU. This could delay the normalization of hyperglycemia. Even though severe hyperglycemia (>180 mg per 100 ml) was reduced, a glucose value within the normal range (80–110 mg per 100 ml) could be achieved in only 49% of the IGC group as shown in Figure 1b. From a methodological point of view, it might be inappropriate to simply count the number of glucose value measurements, as patients with hyperglycemia were monitored more frequently, as defined in this protocol. Furthermore, as the mode of glucose monitoring was quite different between the IGC group and the SGC group, it could be inappropriate to compare the glucose values. A future protocol should include a more appropriate monitoring of glucose level and administration of insulin system that assures the fine tuning of glucose levels within the target range. Finally, there was a possible selection bias that may have affected the results, as this study was not a randomized-control study and there were many nonparticipants. However, the incidence of documented infections in nonparticipants within 100 days after allogeneic HSCT was 42%. Therefore, the reduction in the incidence of documented infections in the IGC group could not simply be explained by other causes such as the selection of antibiotics or catheter management.

With these limitations in mind, we took several steps to improve the quality of the study. First, we carefully matched patients and transplantation characteristics. Second, the IGC strategy was applied prospectively. Third, the low rate of patients who developed clinically significant hypoglycemia should be emphasized. As previously reported, the IGC procedure becomes very difficult in the medical ICU, especially in patients who have sepsis, a high APACHE score or mechanical ventilation.[1,2,22,23] The low rate of hypoglycemia could be because the medical acuity of our patients were relatively mild compared to those of patients in the medical ICU. Moreover, patients undergoing HSCT are younger and might have better β-cell function. The low rate of hypoglycemia could be important for maximizing the benefit of IGC because severe hypoglycemia could be associated with an increased risk of mortality.[23]

The biological plausibility of the intervention should be discussed. The reduction in infectious diseases by IGC may reflect the deleterious effects of hyperglycemia on macrophage or neutrophil function or insulin-induced protective effects on mucosal and skin barriers.[24 27] The improvement of innate immunity could be quite important, especially during the period of granulocytopenia after allogeneic HSCT. The protection of mucosal tissues could reduce bacterial translocation, which might lead to a reduced incidence of sepsis.

In conclusion, our results suggest that prospective IGC reduced the incidences of infectious diseases and organ dysfunction after allogeneic HSCT. To confirm these findings, a larger, prospective randomized-controlled trial is warranted.

Acknowledgements

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Positive impact of maintaining minimal caloric intake above 1.0 × basal energy expenditure on the nutritional status of patients undergoing allogeneic hematopoietic stem cell transplantation

To the Editor: Parenteral nutrition (PN) is frequently required for patients undergoing allogeneic hematopoietic stem cell transplantation (ASCT). However, the recommended dose of PN is associated with hyperglycemia [1,2], which leads to an inferior outcome [1,3]. Body weight (BW) and biochemical indices are used to assess the nutritional status, but these measures are affected by fluid status and inflammation [4]. Therefore, we retrospectively analyzed the values of nutritional variables in a cohort of 112 consecutive adult patients, who received myeloablative ASCT between January 2002 and June 2006. Sixteen patients who died before day 28, developed renal failure or liver failure, or received previous ASCT were excluded. Based on the mean caloric intake from the beginning of the conditioning regimen to day 28 or discharge, the remaining 96 patients were divided into low (n = 67) and high (n = 29) caloric groups [< or ≥ than 1.0 × basal energy expenditure (BEE)]. Patients' characteristics are summarized in Table I. During this period, nutritional support had been left entirely to the individual physicians. Six time periods were considered: (1) before the conditioning regimen, (2) from conditioning to day 0, (3) from days 1 to 7, (4) from days 8 to 14, (5) from days 15 to 21, (6) from days 22 to 28. Biochemical indices including total protein, albumin, cholinesterase, and prealbumin were monitored serially at least once a week.

TABLE I. Patients' Characteristics

Variable	N (%)/ median (range)	
	Low caloric group < 1.0 × BEE n = 67	High caloric group ≥1.0 × BEE n = 29
Age (year)	33 (18–57)	47 (20–55)
Body mass index (kg/m^2)	22.3 (15.2–38.1)	21.0 (15.1–27.2)
Sex		
Male	29 (43)	15 (52)
Female	38 (57)	14 (48)
Conditioning		
TBI-containing	34 (51)	15 (52)
Non-TBI-containing	33 (49)	14 (48)
Stem cell source		
Bone marrow	38 (57)	13 (45)
PBSC	28 (42)	12 (41)
Cord blood	1 (1)	4 (14)

Abbreviations: BEE, basal energy expenditure; TBI, total body irradiation; PBSC, peripheral blood stem cells.

Changes in BW are shown in Fig. 1A: a greater number of patients in the low caloric group lost more than 5% or 10% of their BW compared with the high caloric group (38 vs. 4, $P < 0.001$ and 8 vs 0, $P = 0.1$, respectively). No significant differences were seen for serum albumin, total proteins, cholinesterase, and prealbumin, whereas fasting glucose levels were significantly reduced from days 15 to 28 in the low caloric group (Fig. 1B). The significantly greater weight loss in the low caloric group could be associated with protein loss and organ dysfunction, although changes in fluid status and effects of chronic inflammation should also be considered. The absence of significant differences in biochemical indices between the two groups suggests that these parameters do not directly reflect malnutrition in ASCT patients [5]. Hyperglycemia was observed in patients receiving ≥1.0 × BEE caloric intake. We previously reported that hyperglycemia and neutropenia were associated with an inferior outcome [3]. The results suggest that a minimal caloric intake of >1.0 × BEE is necessary to maintain BW after ASCT, and that the assessment of nutritional status should not rely solely on biochemical indices. However, attention should be paid to the identification and prevention of hyperglycemia in these patients.

Shigeo Fuji[1]
Sung-Won Kim[1]
Takahiro Fukuda[1]
Shigemi Kamiya[2]
Setsuko Kuwahara[2]
Yoichi Takaue[1]

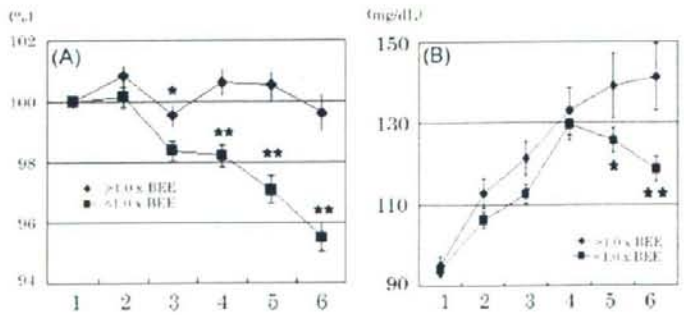

Figure 1. (A) Change in body weight in ASCT (*P 0.05, **P < 0.001). (B) Change in fasting serum glucose level in ASCT (*P 0.06, **P 0.003). The time course was divided into six periods: (1) before the conditioning regimen, (2) from conditioning to day 0, (3) from days 1 to 7, (4) from days 8 to 14, (5) from days 15 to 21, (6) from days 22 to 28.

[1]Department of Hematology and Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan
[2]Division of Nutritional Management, National Cancer Center Hospital, Tokyo, Japan
Published online 8 October 2008 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.21307

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IGF-I treatment of patients with Laron syndrome suppresses serum thrombopoietin levels but does not affect serum erythropoietin

To the Editor: Growth hormone (GH) and insulin-like growth factor I (IGF-I) stimulate the proliferation and differentiation of many cell types including bone marrow cells. IGF-I was shown to stimulate erythropoiesis in in vitro studies [1]. In a previous study, we reported that children with Laron syndrome (LS, OMIM #262500) with congenital IGF-I deficiency responded to IGF-I treatment by an increase of hemoglobin (Hb) and red blood cells (RBC) and a decrease of a high platelet count (PLT) [2]. To investigate whether the effects induced by IGF-I are mediated by erythropoietin (Epo) and thrombopoietin (Tpo), we studied seven patients with LS: three untreated adults (ages: 43, 44, and 52) and four girls aged: 5, 9, 13, and 15 years receiving IGF-I replacement therapy (120–180 μg/kg/day s.c., Fujisawa, Osaka, Japan) for an average period of 9 ± 4 years. The mean age at initiation of therapy was 4.6 ± 3.5 years. Serum Tpo and Epo levels were measured using ELISA kits (Quantikine, R&D Systems, Minneapolis). In the children, before initiation of IGF-I treatment, Tpo levels were above normal for age, m ± SD: 285 ± 189 pg/ml (normal: 15–80 pg/ml). During IGF-I treatment Tpo levels dropped to 36 ± 19 pg/ml ($P = 0.04$). The mean PLT levels before treatment were 334 ± 53 × 10^9/l and decreased to 253 ± 30 × 10^9/l during therapy ($P = 0.04$). In the three untreated adult patients, Tpo serum levels were above normal but the PLT were within the normal limits (Table I). In the IGF-I treated-children, Epo levels did not correlate with the increase of RBC and Hb; and in the untreated adults, Epo levels varied within normal limits (1.0–21.5 mIU/ml). Experimental studies have indicated that the effects of GH on erythropoiesis are mediated by IGF-I of endocrine or paracrine origin [3]. We report for the first time that IGF-I administration reduces the high PLT count in young LS patients concomitantly with serum Tpo levels.

TABLE I. The Effect of IGF-I on Tpo and Platelets

	Before treatment	During treatment	*P* value
LS children			
Tpo (pg/ml)	285 ± 189	36 ± 19	0.04
Platelets (×10^9/l)	334 ± 53	253 ± 30	0.04
Untreated LS adults			
Tpo (pg/ml)	84 ± 60	–	
Platelets (×10^9/l)	240 ± 35	–	

Whether the reduction of Tpo during IGF-I treatment is due to a direct effect of IGF-I on the liver, or whether there exists a negative feedback mechanism between PLT and Tpo synthesis [4], remains to be clarified. The finding that Epo levels do not correlate with the IGF-I induced stimulation of erythropoiesis suggests that this effect is not Epo mediated as was also shown in rats [5] and in children [6]. Recently, it has been suggested that IGF-I secreted by macrophages may directly stimulate erythroblastic islands [7].

ORIT SHEVAH[1]
JOANNE YACOBOVICH[2]
MEIRA ZOLDAN[2]
HANNAH TAMARY[2]
ZVI LARON[1]

[1]Endocrinology and Diabetes Research Unit, Schneider Children's Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Petah-Tikva, Israel
[2]Division of Oncology Hematology, Schneider Children's Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Petah-Tikva, Israel
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Phase III study to evaluate the use of high-dose chemotherapy as consolidation of treatment for high-risk postoperative breast cancer: Japan Clinical Oncology Group study, JCOG 9208

Yutaka Tokuda,[1,12] Tomoo Tajima,[1] Masaru Narabayashi,[3] Kunihiko Takeyama,[3] Toru Watanabe,[3] Takashi Fukutomi,[3] Takaaki Chou,[4] Muneaki Sano,[4] Tadahiko Igarashi,[5] Yasutsuna Sasaki,[5] Michinori Ogura,[6] Shigeto Miura,[6] Shin-ichiro Okamoto,[7] Masami Ogita,[8] Masaharu Kasai,[9] Tadashi Kobayashi,[10] Haruhiko Fukuda,[11] Shigemitsu Takashima,[2] Kensei Tobinai[3] and the members of the Autologous Bone Marrow Transplantation Study Group and the Breast Cancer Study Group of the Japan Clinical Oncology Group (JCOG)

[1]Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193; [2]National Shikoku Cancer Center, 160 Koh, Umemoto-cho, Matsuyama 791-0280; [3]National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [4]Niigata Cancer Center Hospital, 2-15-3 Kawagishi-cho, Chuo-ku, Niigata 951-8566; [5]National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577; [6]Aichi Cancer Center, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681; [7]Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582; [8]Sapporo National Hospital, 2-3-54 Kikusui 4, Shiroishi-ku, Sapporo, Hokkaido 003-0804; [9]Sapporo Hokuyu Hospital, 6-5-1 Hagishisapporo 6, Siroishi-ku, Sapporo, Hokkaido 003-0006; [10]Jikei University School of Medicine, 3-25-8 Nishi Shinbashi, Minato-ku, Tokyo 105-8461; [11]JCOG Data Center, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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A randomized controlled trial was conducted to evaluate the efficacy of high-dose chemotherapy (HDC) as consolidation of the treatment of high-risk postoperative breast cancer. Patients under 56 years of age with stage I to IIIB breast cancer involving 10 or more axillary lymph nodes were eligible. The primary endpoint was relapse-free survival (RFS). Between May 1993 and March 1999, 97 patients were enrolled, and two patients became ineligible. The median age of the 97 patients was 46 years (range 27–55 years), and 72 (74%) were premenopausal. The median number of involved axillary nodes was 16 (range 10–49). All patients had undergone a radical mastectomy. Major characteristics were well balanced between the treatment arms. Forty-eight patients in the standard-dose (STD) arm received six courses of cyclophosphamide, doxorubicin, and 5-fluorouracil followed by tamoxifen. Forty-nine patients were assigned to undergo HDC with cyclophosphamide and thiotepa after six courses of cyclophosphamide, doxorubicin, and 5-fluorouracil followed by tamoxifen; however, 15 of these patients (31%) did not undergo HDC. HDC was well tolerated without any treatment-related mortality. At a median follow-up of 63 months, the 5-year RFS of 47 eligible patients in the STD arm and 48 eligible patients in the HDC arm was 37% and 52% on an intent-to-treat basis, respectively ($P = 0.17$). Five-year overall survival of all randomized patients was 62% for the STD arm and 63% for the HDC arm ($P = 0.78$). Although the prespecified values of the two arms were not so accurate as to allow detection of the observed difference, no advantage of HDC was observed in terms of RFS or overall survival. (*Cancer Sci* 2008; 99: 145–151)

Preclinical studies have suggested that doses of cytotoxic chemotherapy correlate with the cure of cancer patients.[1] Among several kinds of dose-intensification strategies, high-dose chemotherapy (HDC) with autologous hematopoietic stem cell support has been extensively investigated in clinical oncology. In addition, HDC was shown to produce survival advantages in certain types of malignant neoplasms, including relapsed aggressive non-Hodgkin's lymphoma responding to salvage chemotherapy,[2] and untreated multiple myeloma[3,4] in randomized controlled studies.

Adjuvant chemotherapy has been shown to improve relapse-free survival (RFS) and overall survival (OS) in patients with primary breast cancer[5] and dose-intensification was found to be associated with superior outcomes in some populations.[6] However, the prognosis of patients with extensive axillary lymph node involvement is still poor despite conventional-dose adjuvant chemotherapy. Thus, such patients have been considered to be appropriate candidates for clinical trials of HDC.

Several uncontrolled studies have suggested a survival advantage for HDC in the adjuvant treatment of high-risk primary breast cancer with extensive axillary lymph node involvement.[7–11] At the time of writing, 12 adequately conducted randomized controlled trials comparing HDC with standard-dose (STD) or conventional-dose chemotherapy in high-risk postoperative breast cancer patients have been reported.[12–23] In 10 of them, the advantage of HDC was not shown. However, two of them have shown improved RFS from HDC[18,22] and one study has shown an OS benefit.[22] Thus, its role in the treatment of high-risk primary breast cancer is still inconclusive and deserves further attention.

Based on the promising results of uncontrolled phase II trials of HDC for high-risk primary breast cancer, especially those of the Duke series including patients enrolled into the Cancer and Leukemia Group B (CALGB) study 8782, reported by Peters *et al.*[8] phase I/II studies of cyclophosphamide and thiotepa with autologous bone marrow reinfusion[24,25] and our own earlier feasibility study of HDC of cyclophosphamide and thiotepa with autologous stem cell reinfusion against metastatic breast cancer[26] the Japan Clinical Oncology Group (JCOG)[27] conducted a randomized controlled study to evaluate the efficacy of HDC of cyclophosphamide and thiotepa as consolidation of the treatment for high-risk postoperative breast cancer.

Patients and Methods

Patients. The study was designed for women between 15 and 55 years of age with breast cancer, stage I to IIIB, involving 10 or more axillary nodes, histologically confirmed by level II or further dissection. Eligible patients had to have a performance status rating of 0 or 1 according to the Eastern Cooperative Oncology Group (ECOG) criteria.[28] Exclusion criteria were prior chemotherapy, radiotherapy, and endocrine therapy. Patients

[12]To whom correspondence should be addressed. E-mail: tokuda@is.icc.u-tokai.ac.jp

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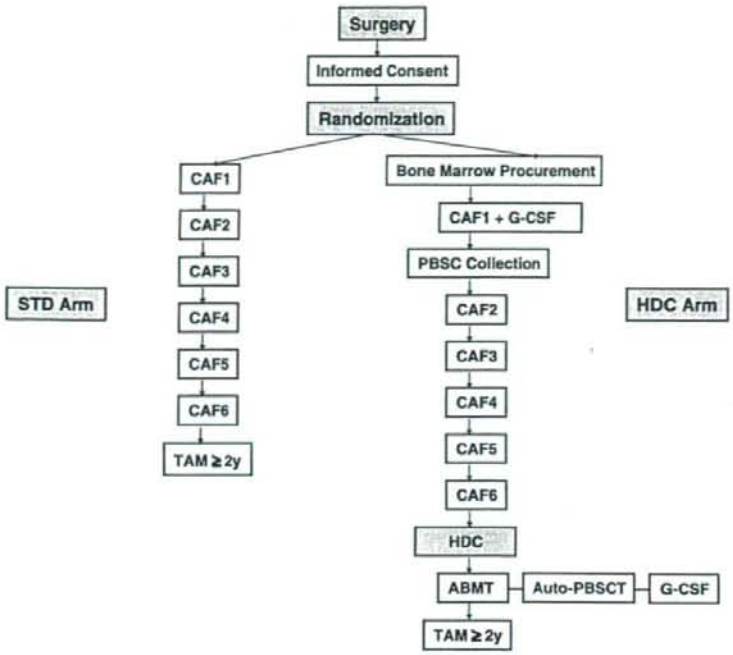

Fig. 1. Trial design of Japan Clinical Oncology Group study, JCOG 9208. ABMT, autologous bone marrow transplantation; CAF, cyclophosphamide, doxorubicin, 5-fluorouracil; G-CSF, granulocyte colony-stimulating factor; HDC, high-dose chemotherapy; PBSC, peripheral blood stem cell; STD, standard-dose; TAM, tamoxifen.

were required to have adequate bone marrow, hepatic, renal, cardiac, and respiratory functions (leukocyte count $\geq 3.5 \times 10^9$/L; hemoglobin ≥10 g/dL; platelet count $\geq 100 \times 10^9$/L; aspartate aminotransferase and alanine aminotransferase ≤4 times the upper normal limit; total bilirubin ≤1.5 times the upper normal limit; blood urea nitrogen and serum creatinine within normal limits; creatinine clearance ≥60 mL/min; no severe cardiac disorder on electrocardiogram; ejection fraction ≥50%; and $PaO_2 \geq 70$ mmHg). Physical examination, chest X-ray, abdominal ultrasound examination, brain computed tomography and a radionuclide bone scan had to be negative for distant metastases. Negative result for bone marrow aspiration or biopsy from the posterior iliac bone was also required.

Patients meeting any one of the following criteria were excluded from the trial: contralateral breast cancer; active concurrent cancer; active peptic ulcer; seropositive for hepatitis B virus surface antigen, hepatitis C virus antibody, or HIV antibody; liver cirrhosis; pulmonary fibrosis or chronic obstructive lung disease; severe psychiatric disorder; diabetes mellitus requiring insulin treatment; uncontrollable hypertension (diastolic pressure ≥110 mmHg); hypercalcemia (serum Ca ≥11 mg/dL); pregnancy or lactation; history of cardiac failure or renal failure; or evidence of concurrent bacterial and fungal infection.

This clinical trial was planned to be conducted at 11 centers belonging to the Autologous Bone Marrow Transplantation Study Group and the Breast Cancer Study Group of JCOG. The JCOG 9208 study protocol and the informed consent document complying with JCOG guidelines and policies were approved by the Clinical Trial Review Committee of JCOG and by the institutional review committee of each participating institution before the start of the study.[27] All patients provided their written or oral consent before the start of the study. Registration involved a telephone call or facsimile from the participating physicians to the JCOG Statistical/Data Center, National Cancer Center, Tokyo, Japan (1991–1997, Statistical Center; 1998–, Data Center).[27] The attending physicians were responsible for submitting periodic data reports on toxicity, relapse, and survival.

Treatment. As shown in Fig. 1, eligible patients were randomly assigned to the STD or HDC arm at the time of enrolment by minimization method to balance the numbers of positive axillary nodes (10–19 or 20–), menopausal status (pre or post) and institution between the arms.

Patients assigned to the STD arm were planned to receive six courses of cyclophosphamide, doxorubicin and 5-fluorouracil (CAF) at 21-day intervals. Each course consisted of intravenous injection with cyclophosphamide 500 mg/m^2, doxorubicin 40 mg/m^2, and 5-fluorouracil 500 mg/m^2. The first course of CAF chemotherapy had to be initiated within 10 weeks after primary surgery.

Patients assigned to the HDC arm underwent bone marrow procurement under general anesthesia before CAF chemotherapy within 9 weeks after primary surgery. Typically, 1 week after primary surgery, they received the first course of CAF together with lenograstim (granulocyte colony-stimulating factor) to collect peripheral blood stem cells (PBSC) as previously described.[29] Lenograstim was given subcutaneously daily from day 8 after CAF chemotherapy until the day of the last leukapheresis. Leukapheresis was carried out once or twice when the leukocyte count increased to greater than 10×10^9/L as described previously.[26] At least 3 weeks after the sixth course of CAF chemotherapy, the patients underwent HDC consisting of cyclophosphamide 2000 mg/m^2/day and thiotepa 200 mg/m^2/day for three consecutive days (days –5 to –3). The doses of cyclophosphamide and thiotepa were determined based on the results of combination phase I/II studies[24,25] and our own feasibility study.[26] Autologous bone marrow and PBSC were thawed and infused on day 0 and 1, respectively. All patients received oral antibiotics, antifungal agents, sulfamethoxazole/trimethoprim and oral acyclovir (200 mg × 5, daily) prophylactically. Irradiated platelet transfusions were given to maintain the platelet count above 20×10^9/L, and irradiated red blood cells were given if necessary. Then 5 μg/kg lenograstim was started on day 2.

Following the above-described therapy, all patients received tamoxifen 20 mg/day for at least 2 years, irrespective of receptor status. Radiation therapy was not planned. All toxicities were

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